EPIDEMIOLOGY AND MOLECULAR CHARACTERIZATION OF *PSEUDOMONAS* SPECIES ON BLUEBERRY PLANTS

by

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B.Sc., University of Manitoba, 2002

Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of
Master of Science

in the
Department of Biological Sciences
Faculty of Science

SIMON FRASER UNIVERSITY

Summer 2012

Approval

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Title of Thesis: ***Epidemiology and molecular characterization of Pseudomonas species on blueberry plants***

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Abstract

Severe bacterial blight occurs periodically in British Columbia's blueberry fields for unknown reasons. *Pseudomonas syringae* pv. *syringae* (Pss) - the putative causal organism, *P. viridiflava*, *P. fluorescens*, *P. tolaasii*, and *P.s*. pathovars *aceris*, *tagetis*, *apii*, and *antirrhini* were isolated from historically blighted fields. Three Pss isolates screened on lilac plantlets were pathogenic. A two-year field study of Pss-inoculated blueberry plants showed that *Pseudomonas* strains are a predominant part of blueberry stem microflora. Internal populations overwintered at low levels (10^1-10^2 CFU/g), but when wounds were present during inoculation 10^6 CFU/g survived. Maximum epiphytic populations reached 10^5 CFU/g in March-April and internal populations reached 10^6-10^7 CFU/g in Feb-May. With wounding, maximum internal populations reached 10^7-10^8 CFU/g as early as Jan-Feb. A colony blot method was developed to identify Pss from diseased tissues. Combined factors including weather, inoculum load and host phenology may contribute to the sporadic nature of bacterial blight.

Keywords: *Pseudomonas syringae*; blueberry; epiphytic survival; overwintering; molecular detection

For my father, who loves his land and all the things that grow there.

Acknowledgements

I would like to thank my advisor, Dr. Zamir Punja, for allowing me to pursue my research interests, for his advice and humour and, above all, for countering my protracted delays with his usual professional alacrity. It has been a pleasure to work in his lab. I also thank Dr. Margo Moore for serving on my advisory committee and for her encouragement and insight. She initiated my renewed and much deeper appreciation of bacteriology. Funding for this research was provided by Agriculture and Agri-Food Canada's Advancing Canadian Agriculture and Agri-Food program through the Investment Agriculture Foundation of British Columbia. Matching funding was provided by the British Columbia Blueberry Council.

I would like to express my gratitude to the blueberry growers of British Columbia for their continued interest and support of research and especially the growers of Delta, Cloverdale and Abbotsford who allowed repeated sampling in their fields. BC blueberry growers continue to produce extremely high quality fruit under increasingly difficult circumstances. Jimmy Gaskin (Gaskin Farms) and Ray Biln (Silver Valley) generously donated potted plants for field experiments and JRT Nurseries donated tissue cultured plantlets. I would like to thank Dr. Janice Elmhirst (Elmhirst Diagnostics) and Grant McMillan (ICMS) for providing space for the field trials. Dr. Diane Cuppels (AAFC Southern Crop Protection and Food Research Centre), Dr. Peter Sholberg (AAFC Pacific Agri-Food Research Centre, Summerland) and Dr. Steven Lindow (UC Berkeley) kindly shared bacterial strains and Lynn Lashuk (Engage Agro) donated BlightBan® A506. Dr. Chris Wendt (NuFarm Agricultural Inc.) donated a sample of Blightban C9-1. Vippen Joshi and Maria Jeffries of the BC Ministry of Agriculture's plant diagnostic lab provided guidance and use of the Biolog microplate reader and software and also shared bacterial strains. Thanks to Grigory Khaskin for assistance with the rotovap and Dr. Dipankar Sen's lab for loan of equipment. SFU Biological Sciences technicians Fan Sozzi-Guo, Cheryl Leonard, Alex Fraser and Leslie Dodd kindly helped with innumerable items in the lab and greenhouse. Thanks to Karen Bedford (AAFC Pacific Agri-Food Research Centre, Summerland) for her suggestions and sharing reprints. SFU (graduate fellowship) provided financial assistance.

This research would not have proceeded without the input and guidance of Dr. Siva Sabaratnam and Mark Sweeney of the BC Ministry of Agriculture. Thank you for

the hours of discussion, suggestions and direction. Mark also obtained a sample of Serenade® on my behalf. I would like to thank Karina Sakalauskas of the BC Blueberry Council for her friendship and her great efforts in being an effective liaison between growers and researchers. Members of the Punja lab past and present, especially Dr. Syama Chatterton, Dr. Owen Wally and Sarah Chen provided invaluable advice, assistance and laughs. I would also like to express my deep appreciation to my partner Ron for his support over the years.

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List of Acronyms

a	Acres
ABC	ATP-binding cassette
ANOVA	Analysis of variance
ARR	Age related resistance
AUDPC	Area under disease progress curve
BC	British Columbia
BCA	Biological control agent
BCMA	British Columbia Ministry of Agriculture
BLAST	Basic local alignment search tool
bp	Basepairs
BUG	Biolog® universal growth medium
cDNA	Complementary DNA
CFU	Colony forming units
CLP	Cyclic lipopeptide
CTAB	Cetyl triethylammonium bromide
cv.	Cultivar
CWDE	Cell wall degrading enzyme
DAMP	Damage-associated molecular patter
DEPC	Diethylpyrocarbonate
dpi	Days post-inoculation
°C	Degrees Celsius
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular polymeric substances
ETI	Effector-triggered immunity
fw	Fresh weight
g	Grams
GLM	General linear model
h	Hours
ha	Hectares
hrc	*Hrp*-conserved
hrp	Hypersensitve response and pathogenicity

HSD	Honestly significant difference test
IAA	Indole-3-acetic acid
INA	Ice nucleation activity
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ITS	Internal transcribed spacer
kb	Kilobasepairs
KB	King's medium B
KBC	King's medium B *Pseudomonas* selective medium
KBC+R	King's medium B *Pseudomonas* selective medium plus rifampicin
L	Liters
LB	Lysogeny broth/Luria broth
LB+Amp	Lysogeny broth plus ampicillin
Log_{10}	Logarithm to base 10
LOPAT	Levan oxidase pectinolytic arginine tobacco
mg	Milligrams
min	Minutes
mL	Milliliter
MLST	Multilocus sequence typing
mm	Millimeter
mM	Millimolar
mN/m	MilliNewton per metre
MOPS	3-(N-morpholino)-propanesulfonic acid
µg	Micrograms
µL	Microliters
µM	Micromolar
NA	Nutrient agar
ng	Nanograms
nm	Nanometers
NRPS	Non-ribosomal peptide synthase
NSA	Nutrient sucrose agar
OD	Optical density
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PDB+cas	Potato dextrose broth plus casamino acids
PDL	Plant diagnostic laboratory
PNW	Pacific Northwest
PR	Pathogenesis-related
Pss	*Pseudomonas syringae* pv. *syringae*
Pss9	Pss blueberry isolate 9
Pss9R	Pss blueberry isolate 9 – rifampicin resistant mutant
PTI	PAMP-triggered immunity
pv.	Pathovar
QS	Quorum sensing
rep-PCR	Repetitive extragenic palindromic sequence polymerase chain reaction
RFLP	Restriction fragment length polymorphism
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT	Room temperature
rt-PCR	Reverse transcriptase polymerase chain reaction
s	Seconds
SDS	Sodium dodecyl sulfate
SDW	Sterile distilled water
SSC	Saline sodium citrate
TBE	Tris-HCl/boric acid/EDTA buffer
TE	Tris-HCl/EDTA
TLC	Thin layer chromatography
T3SS	Type three secretion system
UV	Ultraviolet light
V	Volts
X-gal	5-bromo-4-chloro-3-indoyl- β-D-galactopyranoside

1. Introduction

1.1. Highbush blueberry

The highbush blueberry, *Vaccinium corymbosum* L., is a perennial deciduous woody shrub of the family Ericaceae. This family includes many plants that naturally grow on acid soils such as those found in peat bogs, swamps and bottomlands. Many ericaceous genera are valued for their decorative flowers (rhododendrons, heathers), foliage (wintergreens, Labrador tea), or both (*Pieris*) while others produce edible berries (bearberry, crowberry, salal, huckleberry, lingonberry, bilberry, cranberry). Arguably, the fruit of the blueberry is the most prized and for this reason it has been under cultivation in North America for over 100 years. Original plantations were created by relocating wild plants to marginal lands unsuitable for other crops (Vander Kloet, 1988). Breeding and development of cultivated highbush blueberry varieties (cultivars) began in 1909 in New Jersey (Vander Kloet, 1988).

Approximately 20 North American species of *Vaccinium*, section *Cyanococcus,* produce berries that are blue; however, only a few of these are commercially grown and marketed as “blueberries” while others are wild harvested on a smaller scale. Cultivated blueberries are classified by growth habit as highbush, lowbush (*V. angustifolium*, *V. myrtilloides, V. darrowii*), or rabbiteye (*V. ashei*). Lowbush and rabbiteye are suited to eastern (Nova Scotia, Quebec, Maine) and southern (North Carolina, Georgia, Florida) growing regions, respectively. The natural range of wild highbush blueberry extends from Nova Scotia west to Wisconsin and south to Florida (Eck, 1988). Highbush blueberries have been introduced to the Pacific Northwest, Australia, Europe, South America and China. Northern highbush is the blueberry currently grown in British Columbia, Washington state, Oregon, Michigan and New Jersey. The mild climate of coastal BC and the Fraser Valley allows successful growing of highbush, whose natural northern limit is southern Canada. BC’s native coastal blueberries, *V. ovalifolium* and *V. membranaceum*, are not cultivated (Vander Kloet, 1988).

1.1.1. Economic importance in the Fraser Valley

Highbush blueberry was first introduced to the peat bogs of Lulu Island, Richmond, BC in the 1920's by a farming family from Nova Scotia (van Baalen, 2012). An industry was established rapidly due to favourable factors, including the long bearing season, availability of suitable land, and proximity of a packing plant originally built for potatoes (van Baalen, 2012).

Acreage planted to blueberry has increased dramatically in the Fraser Valley over the last 20 years. Current estimates are 8100 ha (20,000 a) under cultivation producing 40 million kg of fruit annually (BCBC, 2009). Blueberries ranked as BC's 11th top commodity for 2010 behind dairy, farmed salmon, and greenhouse vegetables with total receipts of $59,693,000 (BCMAL, 2010). British Columbia is the foremost producer of blueberries in Canada, with 45.5% of national yield (BCMAL, 2010). Harvested berries supply both retail and wholesale markets with fresh and frozen fruits and juices, plus jam stock, purees, dried berries and concentrates for use in value-added products such as baked goods and confections. Industries associated with berry production, such as packing houses and processors, also benefit from successful crops. International labour migration is supported by BC blueberry growers through the Canadian seasonal agricultural worker program.

1.1.2. Botany

V. corymbosum is a multi-stemmed crown-forming bush whose architecture consists of one major branching bole plus basal shoots that serve as renewal canes, maintaining plant vigour. Young shoots are distinguishable from older canes by girth, lack of recurving, flexibility and appearance of the epidermis. Slender 1-year old shoots, commonly called "whips," are terete (cylindrical) to angular, glaucous (covered by waxy cuticle), smooth to verrucose and coloured green-yellow, turning red in the fall. The shoot cortex is composed of several layers of chlorophyll-containing parenchyma cells interspersed with air canals (Gough, 1994). The young epidermis has many stomata that are converted into corky lenticels as the stem ages (Gough, 1994). In older, woodier canes the cuticle is replaced by grey, scaly bark which easily sloughs off. Older canes recurve to a lesser or greater degree depending on the cultivar. All canes develop lateral shoots and the buds on these shoots "flush" (grow rapidly then cease) to

create the canopy. Each flush is terminated by apical bud abortion leading to “black tip,” a zone of necrosis extending downwards from the shoot apex to the next bud. The affected twig segment is sloughed off in 2-5 weeks (Gough, 1994). Apical abortion releases apical dominance (hormonal suppression of basal growth) and allows further canopy expansion from basipetal buds. Typically there are three flushes of growth in highbush blueberries (Caruso and Ramsdell, 1995). Highbush blueberries can reach heights of 4 m.

Floral buds are found near the tips of fruiting branches, with each bud containing 5-15 individual flowers on a single corymbose inflorescence. Flowers are sympetalous (5 fused petals) with recurved tips, urceolate (urn-) to campanulate (bell-shaped), epigynous (ovary at base of flower), fragrant and generally inverted. The corolla is pink prior to expansion, turning white at maturity. Nectaries are located at the base of the style and attract pollinators. Successfully pollinated flowers produce berries that ripen from green to blue in 4-6 weeks depending on cultivar and environment. Berry colouration is due to anthocyanin development in the pericarp. Delphinidin-, cyanidin-, petunidin-, and malvidin- monogalactoside and malvindin-monoarabinoside are the major anthocyanins (Caruso and Ramsdell, 1995). The blue colour is partly obscured by an epicuticular, glaucous “bloom” and the amount of bloom is cultivar-dependent. Fruits are non-climacteric (respiration and ethylene do not reach a peak during maturation) and must ripen on the bush prior to harvest (Eck, 1988; Gough, 1994). After fruiting is completed, new perennating floral and vegetative buds are initiated in leaf axils and remain dormant until the following spring. Bud type can be distinguished by morphology, with vegetative buds being smaller and having a single apex compared to larger, multi-apex floral buds.

Resumption of normal growth in spring requires a period of low temperatures during dormancy. This period is referred to as the chilling requirement, a specific number of hours below 7.2°C that differs among cultivars and species (Gough, 1994). Northern highbush has a minimum requirement of 800 hours of chilling but 1060-1200 hours produce improved growth by increasing vigourous vegetative bud break (Eck, 1988; Gough, 1994). Floral buds require fewer chilling hours than vegetative buds and are the first to break in spring (Eck, 1988). Some blueberry cultivars can acclimate to

temperatures of -30°C during their midwinter period of maximal cold tolerance (Rowland *et al*., 2008).

1.1.3. Horticulture

Blueberries under cultivation are regularly irrigated, fertilized, and pruned for optimal fruit production. Cultivated blueberry thrives in a pH range of 4.5-5.2 and soil pH is monitored and adjusted as necessary (BCMAL, 2009). Bushes are pruned back to 2.5 m or less. Pruning is also used to open canopies for increased air circulation, light penetration, and ease of harvest by hand-picking or machine. Commercial orchards plant blueberries in blocks, with one cultivar per block but several cultivars per field. Planting of multiple cultivars extends the harvest period when early-, mid-, and late-ripening varieties are used and also allows cross-pollination which can enhance fruit set. Examples of early cultivars are Earliblue and Duke, mid-season cultivars are Draper and Bluecrop, and late ripening cultivars include Rubel and Liberty. Very late cultivars are Elliott and Aurora. Differences between early and very late cultivars allow continuous harvesting of fresh fruits from late June to September (Strik and Finn, 2008). Rented beehives are placed in fields during bloom to assist in pollination.

Bushes are planted approximately 1 m apart in rows of raised beds that allow for increased water drainage. Rows are spaced approximately 3 m apart and are separated either by cover crops (perennial grasses) or mulched bark to minimize weed growth and prevent soil erosion. Rows may be trellised to provide extra support to canes in fields that are machine harvested. Newly planted bushes should be pruned to promote vigourous vegetative and remove generative growth for the first 2 years during establishment.

Quality of nursery stock is assured by clonal propagation of blueberry through rooted cuttings and tissue culture. Stock is also monitored for presence of several viruses that affect blueberry in BC, some of which are quarantine pests. Detection of these viruses requires destruction of affected plants both in the nursery and in growers' fields. Other diseases are not monitored strictly but may be reported by crop scouts and agrologists.

1.1.4. Breeding

Traditionally, blueberry breeders have focused on developing large, flavourful berries. More recent concerns are development of cultivars for different climates, adaptation of plant architecture to machine harvesting, uniform accelerated ripening, and resistance to diseases endemic to specific growing regions, such as *Botryosphaeria* stem canker in North Carolina. Blueberry breeding techniques include natural hybridization, inbreeding, interspecific hybridization, molecular marker assisted breeding (e.g. quantitative trait loci mapping) and tissue culture. To date, there is no sequenced genome available for blueberry; however, a transcriptome based on expressed sequence tags (ESTs) of leaves, fruits and buds exposed to chilling has been published (Rowland *et al*., 2012) and may assist development of cold-hardy varieties.

V. corymbosum is an autotetraploid (2n = 48) and readily hybridizes with other species within the section *Cyanococcus* (Caruso and Ramsdell, 1995; Eck, 1988; Gough, 1994; Vander Kloet, 1988). Breeders have taken advantage of this ability to cross highbush, lowbush and rabbiteye blueberries to achieve opposite goals: increased cold hardiness for northern climates and reduced chilling hour requirements for southern climates (Eck, 1988). Half-high cultivars have also been developed to restrict growth with less pruning. New varieties are released for commercial production only after years of rigorous field trials to assure plant quality and performance.

1.1.5. Pests and management

Cultivated blueberries are subject to many pests common to BC (BCMAL, 2009). Insects such as aphids, midge, leafrollers, spanworms, winter moth larvae, tent caterpillars, fall webworms, sawfly larvae, scale, spotted wing *Drosophila* larvae and weevils feed on leaves, fruits or roots, may transmit viruses, and can carry bacteria and fungal spores between plants (Kluth *et al*., 2002; Nadarash and Stavrinides, 2011; Purcell, 1982), culminating in reduced plant vigour. Birds, snails and nematodes also damage plants, causing wounds that may be colonized by secondary invaders (Marois *et al*., 1992). Fungal diseases such as *Alternaria* fruit rot, *Armillaria* root rot, anthracnose, *Botrytis* fruit rot and blight, *Godronia* canker, mummy berry, *Phomopsis* canker, and *Phytophthora* root and crown rot have been detected in BC (BCMAL, 2009). Viruses in local fields include blueberry scorch carlavirus, blueberry premature fruit drop,

blueberry shock ilavirus and blueberry mosaic virus (BCMAL, 2009). BC Blueberries are affected by two bacterial diseases: crown gall, caused by soil-borne *Agrobacterium tumefaciens* (*Rhizobium radiobacter*), and bacterial blight caused by *Pseudomonas syringae* pathovar (pv.) *syringae* (BCMAL, 2009). Other pests reported on blueberry in other growing regions are either less common or not recorded in BC. These may eventually enter local fields by contamination or natural dispersal or may have escaped detection to date.

Management of the above pests requires carefully timed applications of pesticides plus cultural controls such as pruning for improved air movement in the canopy. Applications of fungicides and insecticides can begin while plants are still dormant in mid-February and continue through the season to October. October is the recommended time for applications of fixed copper to control of bacterial blight occur prior to the onset of fall rains (BCMAL, 2009). Pruning is a post-harvest activity and it is recommended after leaf fall while plants are dormant but it may begin as early as September and continue into February. Pruning is a labour-intensive activity done by hand and therefore is performed as time and personnel are available. The objective of pruning is to remove dead and diseased wood, remove weak or unproductive wood, and stimulate growth of new shoots from the crown (BCMAL, 2009). The benefits of pruning for an open canopy are increased air circulation, which allows quicker drying of leaves and stems to the disadvantage of fungal and bacterial growth, and better penetration of sprayed pesticides.

Fertilizer application can also impact the disease status of plants. High nitrogen has been shown to increase the number of epiphytic phytopathogenic bacteria on leaf surfaces (Balestra and Varvaro, 1997b; Chaudary *et al*., 2009), likely due to concomitant increases in the nitrogen content of leaf exudates. Timing of fertilizer application can also indirectly lead to increased incidence of bacterial blight. Late season application can induce renewed vegetative growth at a time when the plant should cease growth and prepare for dormancy (Deckers and Schoof, 2001). This late flush of growth may not be able to harden off adequately prior to onset of cold weather, leaving it more susceptible to chilling and frost damage and subsequent invasion by bacteria (M. Sweeney, BC Ministry of Agriculture; pers. comm.).

1.2. *Pseudomonas syringae* pv. *syringae* van Hall (Pss)

1.2.1. Description

Pseudomonas syringae pv. *syringae* van Hall (Pss) is an aerobic, chemoorganotrophic Gram negative bacterium of the order γ-Proteobacteria (Palleroni, 1994). Rod-shaped cells are straight or slightly curved, 0.7-1.2 x 1.5 µm, unencapsulated and motile by one or more polar flagella (Palleroni, 1994). Cells most often occur singly but are sometimes in pairs (Hayward and Waterston, 1965; Moore *et al*., 2006). No resting stage is known (Palleroni, 1994). The genome of the fully sequenced Pss strain B728a is 6.1 Mbp (59.2 mol% GC) and consists of a circular chromosome, no plasmids and encodes 5,217 genes (Feil *et al*., 2005); however, other Pss strains are known to carry plasmids (Sundin and Bender, 1993, 1994; Sundin *et al*., 2004) that vary from 50 – 75 kb in size and belong to the pPT23A plasmid family (Sesma *et al*., 2000) first isolated from strains of *P. s.* pv. *tomato*.

Production of diffusible yellow-green fluorescent pigments by Pss can be detected in the laboratory on King's medium B (Palleroni, 1994). This is the peptide-type siderophore, pyoverdin (Cody and Gross, 1987). Some strains of Pss also have been reported to produce achromobactin, a nonfluorescent citrate-based siderophore (Berti and Thomas, 2009) that is highly homologous to achromobactin produced by *Erwinia chyrsanthemi* (Franza *et al*., 2005). Production of both siderophores has been shown to increase epiphytic fitness of Pss (Wensing *et al*., 2010). Newly isolated strains may not produce pigments (Palleroni, 1994).

1.2.2. Optimal growth conditions

Growth is optimum at 25-30°C (Palleroni, 1994), with a range of 0 – 35°C (Hayward and Waterston, 1965; Young *et al*., 1977) and a pH range above 4.5 to neutral (Moore *et al*., 2006; Palleroni, 1994). In assays on both liquid and solid laboratory media, growth of *P. s*. pv. *tomato* was optimal when water potentials ranged from -0.2 to -0.9 MPa, while growth was delayed between -1.4 and -2.3 MPa, and was completely inhibited at -2.7 MPa (Wright and Beattie, 2004). Pss likely has a similar response.

Average growth doubling time of *P. syringae* under laboratory conditions was 1.3 h at 28°C, although growth rates were not significantly different within a range of 23-33°C, signifying an extended temperature plateau (Young *et al*., 1977). Outside this range, doubling times increased to 2.7 h at 16°C, 10.5 h at 5°C, and 22.5 h at 0°C (Young *et al*., 1977). Similar effects of temperature were found in growth studies in the field (Hirano and Upper, 1989). In comparison, *Xanthomonas pruni*, a phytopathogen adapted to warmer growing regions, required 79 h to double at 0°C (Young *et al*., 1977). The ability to maintain metabolism (survive and multiply) at freezing temperatures indicates that *P. syringae* is a psychrotroph, defined as a facultative psychrophile or bacterium whose optimum temperature for growth is above freezing (Morita, 1975; Gounot, 1986). In its natural environment, Pss is subjected to seasonal, daily and hourly fluctuations of temperature. Laboratory growth tests demonstrated that abrupt shifts in temperature cause significantly longer lag phases when the shift is greater (e.g. from 4 to 29°C compared with 11.5 to 29°C), suggesting that physiological adjustments were made (Young *et al*., 1977). Growth over a range of temperatures requires changes in membrane fluidity. This can be accomplished by *cis-trans* isomerization of fatty acids as demonstrated in psycrophilic strains of *P. syringae* (Kiran *et al*., 2005).

Pss has simple nutritional requirements and may utilize many carbon sources such as glucose, succinate, glycerol, fumarate, and pyruvate as well as simple amino acids (Palleroni, 1994) and small peptides. This broad diet may allow colonization of diverse host plants as well as survival in water including rain, snowmelt, streams and rivers (Morris *et al*., 2006, 2008). Although many pseudomonads are common in soil (e.g., *P. putida, P. aeruginosa*), this environment has limited bioavailability of nutrients which may restrict growth, especially in competition with other microbes better adapted to endure periods of starvation (Moore *et al*., 2006) and *P. syringae* does not survive well in soil (Lindow, 1985; Lindow and Panapoulos, 1988; Lindow *et al.*, 1988a). In general, pseudomonads may be better adapted to environments with greater nutrient availability, surviving as opportunistic scavengers or saprophytes (Moore *et al*., 2006). Pss does not accumulate poly-β-hydroxybutyrate (PHB) as an energy storage molecule (Palleroni, 1994).

1.2.3. Taxonomy

Eighteen species in the genus *Pseudomonas* are plant pathogens (Hofte and de Vos, 2006) that together, infect a very wide host range and cause a variety of symptoms including bacterial spots, specks, blights, soft rots, cankers, hyperplasias (galls and scabs) and wilts (Agrios, 1997). *P. syringae* is currently divided into 54 validly described "pathovars," a subspecific designation based on the hosts infected. Host ranges may overlap and therefore pathogenicity on one specific host is insufficient for identification of an isolate (Schroth *et al*., 2006). The pathovar system was developed as an interim expedient to classify distinct pathogens within established taxa such as *P. syringae* (Dye *et al*., 1980; Scroth *et al*., 2006); however, characterization of phytopathogenic pseudomonads remains incomplete. Former pathovars such as pv. *savastanoi* and *avellanae* have been elevated to subspecies (*P*. *s*. subsp. *savastanoi*; Janse, 1982), or species (*P. savastanoi* sp. nov.; Gardan *et al*., 1992; *P*. *avellanae*; Janse *et al*., 1996) while proposals to demote poorly characterized species to pathovars also occur (Dye *et al*., 1980). The current standard for phytopathogenic *Pseudomonas* taxonomy is nucleic acid hybridization of 16S rRNA genes from reference strains and isolates. Similarities of Pss isolates with its reference strain have been reported to range between 95-100% (Schroth *et al*., 2006). Delineation of species requires similarity values of 70% or less (DNA-DNA hybridization) (Wayne *et al*., 1987); however, this value allows for a high level of gene diversity within a putative species (Goris *et al*., 2007) and is the basis for grouping pathovars into genomospecies. Hybridization data can be confirmed with DNA sequence alignments as more bacterial genomes are published.

To date, 9 genomospecies have been distinguished and Pss is categorized in genomospecies 1, which also includes pvs. *aptata*, *lapsa*, *papulans*, *pisi*, *atrofaciens*, *aceris*, *panici*, *dysoxyli*, and *japonica* (Gardan *et al*., 1999). The DNA relatedness of these pathovars to the Pss reference strain ranges from 67% for *aceris* to 87% for *atrofaciens* (Gardan *et al*., 1999) as listed in Table 1-1.

1.2.4. Methods of identification

In 1966, Lelliott *et al*. devised a determinative scheme for identifying phytopathogenic, fluorescent *Pseudomonas* strains. This was based on simple laboratory tests for

Table 1-1. DNA relatedness of Pseudomonas syringae pathovars in genomospecies 1 and other selected pathovars.

Genomospecies[a]	Taxon	CFBP[b] Strain	% DNA-DNA hybridization with Pss strain 1392
1	*P. syringae* pv. *syringae*	1392	100
	P. s. pv. *aptata*	1617	78
	P. s. pv. *lapsa*	1731	82
	P. s. pv. *papulans*	1754	71
	P. s. pv. *pisi*	2105	80
	P. s. pv. *atrofaciens*	2213	87
	P. s. pv. *aceris*	2339	67
	P. s. pv. *panici*	2345	84
	P. s. pv. *dysoxyli*	2356	71
	P. s. pv. *japonica*	2896	90
2	*P. savastanoi*	1670	44
	P. s. pv. *morsprunorum*	2116	53
3	*P. s.* pv. *tomato*	2212	41
	P. s. pv. *morsprunorum*	2351	46
	P. s. pv. *apii*	2103	47
	P. s. pv. *antirrhini*	1620	47
6	*P. viridiflava*	2107	33
7	*P. s.* pv. *tagetis*	1694	50
	P. s. pv. *helianthi*	2067	62

[a] Table adapted from Gardan *et al*. (1999).
[b] Collection Française des Bactéries Phytopathogènes, Angers, France.

biochemical reactions – the LOPAT scheme – which consists of assays for production of *l*evan on sucrose medium, the *o*xidase reaction, *p*ectolytic activity on potato tissue or pectate gel, *a*rginine dihydrolase activity, and the hypersensitive reaction on *t*obacco leaf tissue. Different combinations of positive and negative results point to one or few Pseudomonas species. For example, Pss is $L^+O^-P^-A^-T^+$ while *P. savastanoi* is $L^-O^-P^vA^-T^+$ and *P. cichorii* is $L^-O^+P^-A^-T^+$. Simplified biochemical testing developed by bioMerieux is available as API® and Biotype-100 strips. Further testing is necessary to discriminate between pathovars of a given species.

Automated chemotaxonomic methods also have been developed for efficient, standardized identification of bacteria. Whole cell fatty acid methyl ester (FAME) analysis uses high resolution gas chromatography to profile bacteria based on the composition of over 300 highly conserved fatty acids (Sasser, 2001). Profiling bacteria is also possible through examination of their carbon source utilization. The ability to metabolize an array of carbon sources is tested simultaneously on microtitre plates which are scanned for a positive colorimetric signal that develops as an indicator dye is cleaved from the carbon source during metabolism (Biolog®, 2001). Neither of these methods is recommended for identification to the pathovar level (Braun-Kiewnick and Sands, 2001; Grimont *et al*., 1996).

This issue may be addressed with serological techniques such as the highly specific double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). This method can be used to detect subspecies of *Clavibacter michiganensis* and pathovars of *Xanthomonas campestris*, *X. axonopodis* and *X*. *hortorum* as well as non-culturable *Xylella fastidiosa* (Agdia, 2008; Neogen Europe Ltd., 2007). To date, ELISA kits are commercially available for *P. fluorescens* (Cygnus Technologies, Inc., 2010), *P. s.* pv. *phaseolicola* (Agdia, 2008), *P. s.* pv. *lachrymans*, *P*. *s*. pv. *tomato*, *P*. *s*. pv. *glycinea*, *P*.*s*. pv. *pisi* and Pss (Neogen Europe Ltd., 2007). Kits can be prohibitively expensive for large-scale bacterial identification.

Many molecular tools for bacterial identification have been developed. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for whole cell protein analysis has been used to identify *Pseudomonas* to species and pathovar (Scortichini et al., 2002a; Vancanneyt *et al*., 1996). Genomic fingerprinting methods such as randomly amplified fragment polymorphisms (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), pulsed field gel electrophoresis, repetitive extragenic palindromic sequence PCR (REP-PCR) and multi-locus sequence typing (MLST) have been used to successfully differentiate *P. syringae* pathovars and strains (Bereswill *et al*., 1994; Sisto *et al*., 2007; Sisto *et al*., 2002; Smith *et al*., 1995; Vicente and Roberts, 2007; Sarkar and Guttman, 2004; Bull *et al*., 2011). Standard PCR with primers designed to amplify pathovar-specific genes, such as those involved in toxin or effector synthesis or those encoded on plasmids, is also used to identify pathovars (Sorensen *et al*., 1998; Bultreys and Gheysen, 1999; Lydon and

Patterson, 2001; Takahashi *et al*., 1996, Zaccardelli *et al*., 2005). Colony blots and dot blots based on DNA-DNA hybridization of pathovar-specific sequences are also sensitive assays for simultaneous identification of multiple isolates and have been used to differentiate *P. syringae* pathovars (Denny, 1988; Fanelli *et al*., 2007; Scheck *et al*., 1997). Subtractive hybridization of digested genomic DNA was used to differentiate a specific race of one biovar of *P*. *solanacearum* (Cook and Sequeira, 1991).

Combinations of several techniques may also be useful in profiling pseudomonads from plants. Fluorescent *in situ* hybridization (FISH) using fluorochrome-labelled rRNA probes plus immunofluorescence was demonstrated to be effective in discriminating bacterial subspecies by using flow cytometry to visualize cells (Wallner *et al*., 1996). Westprinting, or western blotting plus immunological fingerprinting, was able to discriminate species of *Pseudomonas* (Tesar *et al*., 1996). Other proposed methods for identifying pseudomonads include siderotyping (siderophore characterization) which may be useful in identification of *P. syringae* strains (Bultreys *et al*., 2001; Meyer, 2000).

Analysis of whole microbial communities is possible via methods like denaturing gradient gel electrophoresis (DGGE) fingerprinting (Heuer and Smalla, 1999; Coenye and Vandamme, 2003), phospholipid fatty acid (PLFA) analysis (Fang *et al* 2000; Johansen and Olsson, 2005), metaproteomics, metagenomics and proteogenomics (Delmotte *et al*., 2009; Hugenholtz and Tyson, 2008). It is now feasible to conduct analyses of plant microbiomes, perhaps to trace differences in communities on different plant organs (flowers, seeds, shoots, roots) or seasonal successions. The use of culture-independent techniques to assess community composition has been shown to reveal a greater diversity of microbial colonizers of plants than is captured with culture-dependent methods (Yang *et al*., 2001).

1.2.5. Habitat

Pseudomonads are ubiquitous, and can be found colonizing soils (Compeau *et al*.,1988; Molina *et al*., 2000; Sorensen and Nybroe, 2004), fresh water (Morris *et al*., 2006, 2008) and salt water (Baumann *et al*., 1983), mushrooms (Gonzalez *et al*., 2009; Goor *et al*., 1986), plants (Agrios, 1997), and occasionally animals (*e*. *g*., *P*. *aeruginosa*, *P*. *mendocina*, *P*. *putrefaciens*, *P*. *fluorescens*, *P*. *putida*) as opportunistic pathogens

(Henry and Speert, 2011; Saeed *et al*., 1987). *P*. *monteilii* and *P*. *mosselii* have been recovered from clinical specimens but have not been implicated in human disease to date (Henry and Speert, 2011). Insects may serve as vectors for *P*. *chlororaphis* (Snyder *et al*., 1998), *P*. *syringae* (Groth *et al*., 1995) and potentially other pseudomonads, but they also succumb to pathogenic species such as *P*. *entomophila* (Vodovar *et al*., 2005, 2006). Increasing evidence suggests that *P*. *syringae* can act as a primary pathogen of its insect vectors such as pea aphids (Nadarash and Stavrinides, 2011). Pseudomonads also have been isolated from processed foods and are involved in spoilage that mostly affects dairy and meat products by production of hydrolytic enzymes (Rajmohan *et al*., 2002) and siderophores to outcompete other bacteria (Gram *et al*., 2002). They are one of many metabolically active bioparticles found in high atmosphere clouds (Amato *et al*., 2005; Vaitilingom *et al*., 2010) where they play a role in the water cycle (Morris *et al*., 2008). Pseudomonads are also associated with low atmosphere fogs, attaining concentrations of 10^4 CFU/m^3 in the Po Valley of Italy (Fuzzi *et al*., 1997). Psychrophilic species such as *P*. *antarctica*, *P*. *meridiana* and *P*. *proteolytica* (Reddy *et al*., 2004) and strains of *P*. *putida*, *P*. *fluorescens*, and *P*. *syringae* (Shivaji *et al*., 1989) have been isolated from Antarctic lakes and soils while moderately thermotolerant species such as *P*. *duriflava* (Liu *et al*., 2008) and *P*. *xinjiangensis* (Liu *et al*., 2009) have been isolated from desert soils.

P. syringae is found most commonly in association with plants but is not an obligate parasite. As with the environmental human pathogens *Legionella*, *Vibrio*, and *Mycobacterium*, *P*. *syringae* has adaptations for survival in aquatic environments including pristine rivers, streams, snow, rain and lakes, often far removed from agricultural sites (Morris *et al*., 2006, 2008). *P*. *syringae* has been implicated in bio-precipitation through its ability to nucleate snow and rain droplets (Christener *et al*., 2008; Ahern *et al*., 2007). This may allow cells in the atmosphere to return to Earth, dispersing over plant canopies on a global (Christener *et al*., 2008) or local scale (Constantinidou *et al*., 1990). *P*. *syringae* strain 31a has been fermented industrially and formulated as the commercial product Snomax® Snow Inducer for use by winter resorts as a natural snowmaking additive active at a relatively warm temperature of 27°F (-2.8°C) (Annecy LLC, 2012).

1.2.6. Host plant species

1.2.6.1. Woody host plants

Pathovars of *P*. *syringae* are endemic on plants and affect almost all cultivated crops grown worldwide (Collmer *et al*., 2009). The diseases are typified by necrotic lesions on susceptible tissues, producing a variety of symptoms. Specific reports of Pss symptoms on woody hosts are listed in Table 1-2.

In the Pacific Northwest (PNW – regions of Oregon, Washington state and British Columbia west of the Cascade and Coast mountain ranges), *P*. *syringae* pathovars *apii*, *berberidis*, *coriandricola*, *coronafaciens*, *coryi* (synonym of *P. avellanae*), *delphinii*, *lachrymans*, *maculicola*, *mori*, *morsprunorum*, *phaseolicola*, *philadelphi*, *pisi* and *viburni* have been reported on horticultural and crop plants (Pscheidt and Ocamb, 2008; Scheck *et al*., 1996). Woody hosts in this region are affected by *P.s*. pvs. *berberidis*, *mori*, *morsprunorum*, *philadelphi*, and *viburni*, which typically produce only leaf spot symptoms (Pscheidt and Ocamb, 2008). However, *P. s*. pv. *mori* can cause necrosis and dieback on young shoots of mulberry, pv. *morsprunorum* has been known to cause necrotic streaking on 1-year old stems of roses, pv. *philadelphi* can affect terminal shoots of mock orange and pv. *viburni* occasionally causes stem lesions and shoot dieback when viburnum is heavily affected (Pscheidt and Ocamb, 2008). *P. s*. pv. *morsprunorum* is a major pathogen of cherry trees in Ontario (Allen and Dirks, 1978), Michigan (Latorre and Jones, 1979; Renick *et al*., 2008) and England (Crosse, 1957; Crosse, 1959; Crosse1963) but is not reported as a major pathogen in BC.

Pss has been reported on woody ornamental and orchard species such as sweet cherry, flowering cherry, cherry laurel, cotoneaster, *Euonymus*, *Forsythia*, lilac, *Magnolia*, maple, mock orange, pear, poplar, willow (Pscheidt and Ocamb, 2008), ash, dogwood, *Eucalyptus*, flowering almond, hazelnut, linden and tree peony (Canfield *et al*., 1986) in the PNW. Many ericaceous plants are common horticultural specimens (*e*. *g*. rhododendron and azalea, heaths and heathers, bearberry, madrone, *Pieris*,

Table 1-2. Woody hosts and disease symptoms associated with Pseudomonas syringae pv. syringae.

Host	Symptoms	Reference(s)
Mango	Apical necrosis	Cazorla *et al*. (1998); Golzar and Crother (2008)
Hazelnut	Decline	Scortichini *et al*. (2002b)
Citrus	Blast, black pit, bacterial brown spot	Shigeta and Nakata (1995); Timmer *et al*. (2000)
Persimmon	Leaf necrosis and twig dieback	Scortichini *et al*. (1998)
Pear	Bud and blossom blast	Mansvelt and Hattingh (1987, 1987a, 1987b); Qiu *et al*. (2008); Wen (2008); Whitesides and Spotts (1991)
Apple	Bark blister, fruit spur blight, bud blight	Sholberg and Bedford (1997); Dhanvantari (1969); Mansvelt and Hattingh (1986, 1987, 1989); Hatting *et al*. (1989); Burr and Katz (1984)
Cherry	Stem canker and bud blight	Foulkes and Lloyd (1980); Hatting *et al*. (1989); Latorre *et al*. (1985); Li and Sholberg (1992)
Peach	Blossom blast, stem canker, and dieback, short life disease	Endert and Ritchie (1984); Little *et al*. (1998); Ritchie and Clayton (1981)
Plum	Blossom blast, stem canker, and dieback	Hinrichs-Berger (2004)
Apricot	Blossom blast, stem canker, and dieback	Hattingh *et al.* (1989)
Kiwi (vine)	Floral bud necrosis and stem canker	Balestra and Varvaro (1997a); Opgenorth *et al*. (1983); Rossetti and Balestra, 2008

wintergreen, mountain laurel) but there has only been one report of *P. cichorri* as a leafspot pathogen of rhododendron (Uddin and McCarter, 1996). Pss on *Arbutus pavarii* (Ericaceae) has been reported in Libya as a leaf spot pathogen but did not affect woody tissues (Boubaker *et al*., 2009). No investigations have been conducted to explore why related genera are generally less susceptible than blueberry.

It has been found that newly sequenced *Pseudomonas* pathogens of woody hosts have adaptations common to soil- and root-colonizing bacteria that are not found in other phytopathogenic *Pseudomonas* strains. *P. syringae* pv. *aesculi*, causal agent of horse chestnut bleeding canker, and *P*. *savastanoi* pv. *savastanoi*, causal agent of olive galls, both carry a genomic island encoding enzymes necessary for metabolism of recalcitrant phenolic compounds common in both soils and wood (Green *et al*., 2010; Rodriguez-

Palenzuela *et al*., 2010). *P*. *syringae* pv. *actinidiae* also has been reported to carry genes encoding wood-degrading enzymes although it infects kiwi, a woody vine (Scortichini *et al*., 2012) It is not known whether Pss isolates from woody hosts share this feature since only isolates from herbaceous hosts (bean) have been sequenced to date.

P. syringae was first reported on blueberry as a stem canker agent in Oregon in the 1950's (Stace-Smith *et al.*, 1953) and was later confirmed to cause stem necrosis and bud blight with symptoms appearing in the spring in PNW fields (Canfield *et al*., 1986). To date, two other reports of Pss on blueberry have come from Chile (Guerrero and Lobos, 1989) and Australia (Wilson and Sampson, 1984). In spite of the dearth of published research, bacterial blight due to Pss is listed in the compendium of blueberry diseases (Caruso and Ramsdell, 1995).

1.2.6.2. Infection process

Pss is considered an opportunistic pathogen because it can enter plant tissues only through wounds (caused by insect and mechanical damage) or natural openings. Phytopathogenic bacteria that occupy the phylloplane commonly use stomata (air-exchange pores in leaf surfaces), hydathodes (water secretion pores), nectarthodes (nectar secretion pores), lenticels (air-exchange pores in stem periderm and fruit pericarp), and wounds such as growth cracks, broken trichomes (leaf hairs), and the excision scars from leaf and bud scale dehiscence to access the apoplast (air-filled intercellular spaces; reviewed in Huang, 1986). Wounds from pruning have been associated with the formation of large cankers in peach trees (Kennelly *et al*., 2007).

Examinations of plant surfaces by SEM (Mansvelt *et al*., 1987a, 1987b, 1989) or epifluorescence microscopy (Monier and Lindow, 2004) have shown Pss aggregations around natural openings and trichomes. As a motile bacterium, Pss can move through water films towards these sites when attracted by either aerotaxis or chemotaxis (Huang, 1986). Alternatively, bacteria landing on plant surfaces may preferentially adhere to these sites, enhancing survival (Mansvelt and Hattingh, 1987a). Pathogenic bacteria have been shown to be more competent than nonpathogenic bacteria at populating these sites, especially under environmental stress such as desiccation (Wilson *et al*.,

1999). Once inside the plant, bacteria can survive and multiply rapidly in the apoplast using nutrients and moisture released from the plant cell cytosol (Alfano and Collmer, 1996; Melotto *et al*., 2008). Systemic infections do not occur since Pss remains in the leaf and stem mesophyll and does not colonize the vascular tissues. However, in instances of severe cankering or wounding, vascular tissues can be affected and killed.

Once bacterial multiplication has begun to produce sufficient numbers of cells, the site is established as an infection court from which disease initiates and spreads within the plant (Hirano and Upper, 2000). The titer required to initiate disease may depend on the host-pathogen system plus environmental conditions. Varieties of a single crop may display a range of resistance to the same pathogen. For example, in the well-studied interaction of *Phaseolus vulgaris* (common bean) and *P*. *syringae*, susceptible varieties develop necrotic lesions while resistant varieties exhibit the hypersensitive response reaction (Daub and Hagedorn, 1980). This plant defense response is described in further detail in section 1.3.2.

P. *syringae* is a hemibiotroph (Collmer *et al*., 2009; Glazebrook, 2005; Thaler *et al*., 2004), a pathogen which has characteristics of both a biotroph, which obtains nutrients from live host cells, and a necrotroph, which obtains nutrients from dead or dying host cells. This differs from latent infection since the pathogen is proliferating and actively suppressing host defenses during the biotrophic stage rather than lying dormant (Lee and Rose, 2010). The phases of hemibiotrophy depend on the stage of infection, with the asymptomatic, biotrophic lifestyle occurring in early stages of infections and the symptomatic, necrotrophic lifestyle occurring in later stages. Mature *P*. *syringae* biofilms confer resistance to antibiotics (Tomihama *et al*., 2007) as well as environmental stresses and enhance communication between cells (Tolker-Neilsen and Molin, 2004).

1.2.7. Virulence factors

Pss has a repertoire of virulence factors that contribute to its survival, spread, and pathogenicity. Necrotrophic populations within hosts secrete toxins, polysaccharides, phytohormones, and effectors that all contribute to disease development.

1.2.7.1. Toxins

Plant-associated pseudomonads produce many phytotoxic peptide and polyketide compounds that produce chlorotic and necrotic symptoms in host tissues (Bender *et al*., 1999; Volksch and Weingart, 1998). Toxin activity is not host-specific (Raaijmakers *et al*, 2006; Volksch and Weingart, 1998). Phytotoxins are not strictly required for pathogenicity of *P*. *syringae*; however they do enhance virulence and are associated with increased disease severity but not increased population size (Xu and Gross, 1988; Scholz-Schroder *et al*., 2001).

Syringomycin is a toxin produced by both pathogenic and nonpathogenic strains of Pss (Gross and DeVay, 1977; Quigley and Gross, 1994) and by *P*. *s*. pv. *aptata*, pv. *atrofaciens*, and *P*. *fuscovaginae* (Bender *et al*., 1999). It is a cyclic lipopeptide (CLP) composed of a polar nonapeptide head with a fatty acid tail (Segre *et al*., 1989). The head is circularized by formation of a lactone ring between the N-terminal serine and the C-terminal threonine (Segre *et al*., 1989). Length of the tail may vary (10, 12, or 14 C atoms), producing three forms, two of which are more active and abundant (Segre *et al*., 1989). Some unusual features of this toxin are the inclusion of the amino acids 2,3-dehydroaminobutric acid (Dhb), 3-hydroxyaspartic acid, 4-chlorothreonine ((4-Cl)Thre), and D-isomers of serine and 2,4-diaminobutyirc acid (Dab; Bender *et al*., 1999). Toxin activity declines as the chlorine is substituted with bromine or the threonine is unhalogenated (Grgurina *et al*., 1993). Presence of uncommon amino acid indicates that the toxin is not synthesized by ribosomes. Instead, large non-ribosomal peptide synthase (NRPS) complexes use a non-RNA-dependent multicarrier thiotemplate mechanism to synthesize syringomycin and its related toxins (Zhang *et al*., 1995; Nybroe and Sorensen, 2004), comparable to synthesis of gramicidin S, a cyclic oligopeptide antibiotic produced by *Bacillus brevis* (Matteo *et al*., 1976) and other microbial antibiotics. Such peptides are synthesized from a large variety of proteinogenic and non-proteinogenic amino acids including glycosylated and N-methylated residues, heterocyclic elements and D-amino acids (Grunewald and Marahiel, 2006).

The related Pss CLPs include syringotoxin, syringostatin, pseudomycin, and eomycin (Nybroe and Sorensen, 2004). Although there are some differences in amino acid sequences of these CLPs, they maintain similar biological activity (Sorensen *et al*.,

1996). A second class of Pss CLP is the syringopeptins. These toxins are larger molecules with different amino acid composition (Ballio *et al*., 1991). The peptide moiety consists of 22 or 25 amino acid residues attached to a 10-carbon or 12-carbon fatty acid tail, resulting in five different forms synthesized by different Pss strains (Bender and Scholz-Schroeder, 2004). They share features of syringomycins such as the nonapeptide head, presence of unusual amino acids including Dhb, Dab, (4-Cl)Thre, ornithine, and D-isomers (Ballio *et al*., 1991, 1995) and synthesis by NRPS. Many of the other 13 or 16 amino acids of syringopeptin are hydrophobic (Ballio *et al*., 1995). The amphipathic nature of CLPs allows insertion of hydrophobic tails into plant plasma membranes, thereby forming pores and resulting in cytolysis (Hutchison and Gross, 1997; Hutchison *et al*., 1995; Carpaneto *et al*., 2002). The inserted monomers can aggregate to form larger complexes with larger water-filled pores of up to 1.7 nm diameter (Dalla Serra *et al*., 1999). Monomers may co-ordinate to synchronize opening and closing of the pore (Kaulin *et al*., 1998). Syringomycin and syringopeptin can be produced by a single Pss strain simultaneously but have not been found to associate in single pore complexes (Hutchison and Gross, 1997).

Syringomycin is a potent biological surface-active (biosurfactant) compound that can lower the interfacial tension of pure water from 73 mN/m to 31 mN/m (Hutchison *et al*., 1995). Biosurfactant activity of *Pseudomonas* CLPs aids in the colonization of host tissues by augmenting wettability of plant surfaces, facilitating movement of bacteria and enhancing penetration of secreted products to the site of activity (Lindow and Brandl, 2003; Raaijmakers *et al*., 2006). CLPs are also antimicrobial and can inhibit growth and spore germination of various fungi (Lavermicocca *et al*., 1997) using the pore-forming mechanism and interaction with sphingolipids of the fungal membrane (Stock *et al*., 2000). Assays of fungal antagonism *in vitro* are quick methods for assessing production of syringomycin and related CLPs (Brancato and Golding, 1953; Sinden et al 1971; Vassiliev et al 1996). Syringomycin and syringopeptin have been implicated in stomatal closure on detached leaf and epidermal strip experiments (Di Giorgio *et al*., 1996). This may improve the substomatal chamber as a protected site for bacterial colonization once Pss has gained entry (Di Giorgio *et al*., 1996).

Genes encoding the regulation, synthesis and secretion of syringomycin (*syr*) and syringopeptin (*syp*) are clustered and adjacent to each other as a genomic island on the

chromosome (Zhang *et al*., 1995, 1997; Scholz-Schroeder *et al*., 2003; Quigley and Gross, 1994; Raaijmakers *et al*., 2006). Although syringomycin and syringopeptin are synthesized by separate pathways, they may share regulatory and secretory mechanisms (Grgurina *et al*., 1996). The *syrD* locus lies between the *syr* and *syp* clusters (Scholz-Schroeder *et al*., 2001) and was predicted to encode an ATP-binding cassette (ABC) transporter (Quigley *et al*., 1993). Additional studies have shown that a three-component resistance-nodulation-cell division (RND) type efflux system is involved in secretion (Kang and Gross, 2005).

Production of Pss CLPs is triggered in response to plant signal molecules (Mo and Gross, 1991b; Mo *et al*., 1995; Quigley and Gross, 1994; Wang *et al*., 2006). Phenolic glycosides, but not aglycone derivatives, induced *syrB* expression and syringomycin production (Mo and Gross, 1991b). Expression could be enhanced further by the addition of common plant sugars including D-fructose, D-mannose and sucrose which seem to increase sensitivity to the phenolic signals (Mo and Gross, 1991b; Quigley and Gross, 1994). Syringopeptin expression is upregulated by the same plant compounds (Quigley and Gross, 1994; Wang *et al*., 2006). Some strains of Pss would only produce detectable amounts of CLPs in the presence of plant compounds *in vitro* (Quigley and Gross, 1994), suggesting that Pss strains previously reported not to produce CLPs merely lacked the proper additives in the growth medium. Blueberry (Prior *et al*., 1998; Riihinen *et al*., 2008), pear (Durkee *et al.,* 1968) and cherry (Mo *et al.,* 1995) tissues are rich in the phenolic β-glucosides quercetin, kaempferol and arbutin. Plant molecule signals are transmitted to the *syr-syp* biosynthesis mechanism by the global regulators GacS-GacA (Wang et al., 2006). SalA, an element of the *gacS-gacA* regulon binds to SyrF, an intermediary regulator of the *syrB* operon (Wang *et al*., 2006).

Other factors can influence CLP expression in Pss. Iron concentration positively regulates *syrB* expression and toxin production (Mo and Gross, 1991b). Chlorination of the threonine residue is mediated by SyrB2, a nonheme Fe^{II} α-ketoglutarate-dependent halogenase (Vaillancourt *et al*., 2005). SyrB2 coordinates iron, α-ketoglutarate and a chloride ion in a facial triad (Blasiak *et al*., 2006). Inorganic phosphate concentrations greater than 1 mM repress syringomycin production, similar to the downregulation of antibiotic synthesis by other bacteria (Bender *et al*., 1999). Finally, arginine auxotrophs cannot synthesize syringomycin (Lu *et al*., 2003). The syrA locus encodes N-acetyl-

glutamate synthetase which catalyzes the first step in arginine synthesis from L-glutamate (Cunin *et al*., 1986). Arginine is one of the 9 amino acids of the cyclic peptide head of syringomycin (Segre *et al*., 1989).

1.2.7.2. Extracellular polysaccharides

Extracellular polymeric substances (EPS) are produced and secreted by many diverse bacterial genera such as *Rhizobium* (Gonzalez *et al*., 1996; Battisti *et al.,* 1992), *Escherichia* (Danese *et al*., 2000), *Streptococcus* (Folkenberg *et al*., 2006; Koo *et al*., 2010), *Erwinia* (Torres-Cabassa, 1987), *Pantoea* (Morohoshi *et al*., 2011), *Vibrio* (Ali *et al.,* 2002), *Xanthomonas* (Guzzo *et al.,* 1993), *Xylella* (Rodrigues da Silva, 2001; Roper *et al*., 2007) and *Pseudomonas*. Exopolysaccharides are carbohydrate polymers that may carry noncarbohydrate substituents (Whitfield, 1988). In plant pathogens, the functions of EPSs include formation of capsule layers bound to the cells, components of slimes and biofilms and contribution to virulence and/or environmental fitness (Leigh and Coplin, 1992). EPSs contribute important properties for survival in the environment, namely hygroscopy and viscosity (Leigh and Coplin, 1992). Two major exopolysaccharides produced by *P*. *syringae* are alginate and levan (Laue *et al*., 2006).

Alginate is an unbranched heteropolymer of β-1,4-linked D-mannuronic acid and L-guluronic acid residues (Jain and Ohman, 2004; McGroarty, 1998). The residues are arranged in repeating blocks as monomers or heteropolymers (Jain and Ohman, 2004). Alginate was first isolated from kelp and is a major structural component of brown algae useful in many industrial applications (Draget *et al*., 2005). Bacteria that produce alginate include *Azotobacter vinlandii* (Gorin and Spencer, 1966), *P*. *aeruginosa* (Linker and Jones, 1966), *P*. *fluorescens*, *P*. *mendocina*, *P*. *putida* (Govan *et al.*, 1981) and Pss (Kidambi *et al*., 1995). Many other phytopathogenic *Pseudomonas* strains differentially produce alginate in the laboratory on various synthetic media (Fett *et al*., 1986). Alginates from all sources are composed of the same mannuronic and guluronic subunits; however, the arrangement of these monomers and subsequent configuration of the end polysaccharide may differ between organisms (Jain and Ohman, 2004).

Regulation of alginate synthesis is best studied in *P*. *aeruginosa* given its clinical significance for immunosuppressed patients but shares homology with Pss (Fakhr *et al*., 1999; Penaloza-Vazquez *et al*., 1997). Briefly, the alternative sigma factor encoded by

algT and the response regulator AlgR1 are required for transcription of genes in the alginate biosynthesis pathway and expression of the mucoid phenotype for both bacteria (Fahkr *et al*., 1999). Whereas this phenotype is induced by high osmolarity, dehydration and oxidative stress in *P*. *aeruginosa* (Jain and Ohman, 2004) and *P. putida* (Chang *et al*., 2007), an additional environmental signal for alginate production in Pss is copper (Kidambi *et al*., 1995), the active ingredient in common formulations of agricultural bactericides. Other heavy metals did not induce alginate synthesis (Kidambi *et al*., 1999).

The role of alginate in environmental fitness of Pss has been attributed to enhanced colonization and dissemination of the bacteria within the host (Leigh and Coplin, 1992; Yu *et al*., 1999). Alginate may aid in adhesion to plant surfaces (Fett *et al*., 1986; Laue *et al*., 2006) and avoidance of plant defenses (Leigh and Coplin, 1992). Studies on other alginate-producing pseudomonads have demonstrated that this EPS is important for survival under water-limiting conditions. In *P*. *putida*, alginate synthesis was induced by matric stress conditions, altered biofilm ultrastructure and thereby increased tolerance to desiccation by contributing to a hydrated microenvironment (Chang *et al*., 2007). Alginate has been shown to chelate copper ions (Kazy *et al*., 2002) and this would greatly increase fitness in regions with frequent applications of copper pesticides. For both *P*. *solanacearum* and Pss, alginate deficient mutants are less virulent on host plants (Kao *et al*., 1992; Yu *et al*., 1999).

Levan is a high molecular weight homopolymer of β-2,6-linked D-fructofuranosyl with extensive β-2,1-branching (Laue *et al*., 2006). It is synthesized by an extracellular enzyme, levansucrase, which cleaves, then links, fructosyl moieties from sucrose, releasing glucose (Li and Ullrich, 2001). Levan is produced by Gram-positive and -negative bacteria including *Leuconostoc mesenteroides* (Holt and Cote, 1998)*, Bacillus subtilis* (Shih *et al.,* 2005), *Streptococcus mutans* (Simms *et al*., 1990), *Zymomonas mobilis* (Alegre *et al*., 2005), *Gluconacetobacter diazotrophicus* (Hernandez *et al.,* 1995), *Erwinia amylovora* (Blake *et al.,* 1982), *P*. *viridiflava* strains (Gonzalez *et al*., 2003), *P*. *savastanoi* strains (Marchi *et al*., 2005) and pathovars of *P*. *syringae*.

Levansucrase is encoded by two functional genes in *P*. *syringae* pv. *glycinea*, one chromosomal (*lscC*) and the other (*lscB*) on an indigenous plasmid (Li and Ullrich,

2001). Although there are probably many levansucrase isoenzymes in the *P. syringae* complex (Li and Ullrich, 2001), the enzyme seems conserved among levan-producing Gram negative bacteria (Hettwer *et al*., 1995). Transcription of both *lscB* and *lscC* was found to be thermally regulated, with increased expression and secretion at 18°C vs. 28°C (Li *et al*., 2006). Levansucrase expression occurred mainly during the exponential growth phase of planktonic and sessile *P. syringae* laboratory cultures (Laue *et al*., 2006). Secretion probably requires a two step, signal peptide-independent pathway since it otherwise would accumulate in the periplasm (Li and Ullrich, 2001). In *Gluconacetobacter diazotrophicus,* a type 2 secretion system translocates levansucrase (Arrieta et al., 2004) but secretion in *P. syringae* does not match any known Gram negative pathways (Li *et al*., 2006).

Levan may prevent intimate contact of bacteria and host cell walls by reducing viscosity when mixed with pectin, a plant cell wall component (Kasapis and Moore, 1994). This may function in pathogenesis by masking the pathogen and preventing detection by the host (Hettwer *et al*., 1995; Kasapis and Moore, 1994). Levan is also a structural component of *P. syringae* biofilms, accumulates in voids within the film and may act as a source of stored nutrients for late stages of film development (Laue *et al*., 2006).

1.2.7.3. Indole-3-acetic acid synthesis

Indole-3-acetic acid (IAA) is an important phytohormone involved in normal plant growth and development. Synthesis of IAA by phytopathogenic bacteria is major virulence factor for *P. savastanoi* pv. *savastanoi* (inducing knot formation in olive, oleander, ash and other host trees), *Agrobacterium tumefaciens* (crown gall formation) and *A. rhizogenes* (hairy root disease) (Agrios, 1997). IAA is commonly produced by *P. syringae* pathovars (Glickmann *et al*., 1998) including Pss (Fett *et al*., 1987). The role of IAA is not clear for Pss since it does not induce proliferation of host cells as *P. savastanoi* does. It has been suggested that IAA may increase epiphytic fitness or aid in toxin production (Mazzola and White, 1994) or it may be involved in suppressing the hypersensitive response (Robinette and Matthyse, 1990), a host response whereby localized programmed cell death is intended to restrict the growth and spread of a pathogen within the plant. Other proposed functions of IAA from phytopathogenic bacteria that do not cause hypertrophy include modification of the proton gradient across

the host plasmalemma to promote solute uptake, increases in respiration, protein synthesis, and enzymatic activity (Fett *et al*., 1987). IAA may also trigger production of ethylene, a plant hormone involved in flower and leaf senescence and dehiscence, fruit ripening and dehiscence, release from dormancy and other physiological responses (Raven *et al*., 1992). Tissue senescence and dehiscence would provide bacteria with nutrients and entry points into the plant. Exogenously applied IAA can antagonise the effect of a third plant hormone, abscisic acid (ABA), which is involved in stomatal closure (Acharya and Assmann, 2009; Tanaka *et al*., 2006). Production of IAA may therefore enhance the ability of Pss to use stomata as entry points.

1.2.7.4. Type three secrection system and effectors

Pseudomonads are one of many bacterial genera that possess a type three secretion system (T3SS). The T3SS has been described as a molecular syringe used by the bacteria to inject effectors (virulence proteins) directly into host cells (Buttner and He, 2009). The T3SS is encoded by the *hrp* (*h*ypersensitive *r*esponse and *p*athogenicity) and *hrc* (*HR*conserved) gene cluster (Collmer *et al*, 2000) and its construction is homologous to flagellar biosynthetic mechanisms (Gauthier *et al*., 2003). The *hrp* and *hrc* genes also encode effectors and are essential for pathogenicity (Mohr *et al*., 2008; Collmer *et al*., 2000). It has been reported that naturally occurring non-pathogenic isolates of *P. syringae* lack the T3SS and effector genes (Mohr *et al*., 2008).When transformed with a plasmid encoding the T3SS and a sinlge effector gene, the ability of this strain to grow *in planta* increased ten-fold (Morh *et al*, 2008).

Collectively, bacterial effectors contribute to pathogenesis by modulating host hormone expression, suppressing host miRNA pathways and by silencing plant defense responses (Cunnac *et al*., 2009). *Hop* (*H*rp-dependent *o*uter *p*rotein) genes and *avi*rulence genes (*avr*) within the *hrp-hrc* cluster encode the known *P. syringae* effector proteins listed in Table 1-3. As reviewed in Block *et al*. (2008), specific activities of bacterial effectors include kinase inhibition (*P*. *s*. pv. *tomato* AvrPto), E3 ubiquitination (*P*. *s*. pv. *tomato* AvrPtoB) and cysteine proteases (*P*. *s*. pv. *tomato* AvrRpt2; *P*. *s*. pv. *phaseolicola* race 3 AvrPphB) targeting host receptors, phosphorylation targeting host receptors (*P*. *s*. pv. *glycinea* race 0 AvrB and AvrRpm1), transcription activator-like targeting host *R* gene products of unknown function (*Xanthomonas oryzae* pv. *oryzae*

Table 1-3. Currently known effectors from P. syringae pv. syringae.

Effector	Function/Activity	Reference(s)
AvrB3	Elicited cell death in tobacco	Alfano *et al*. (2000); Charity *et al*. (2003)
AvrE1	unknown	Huang *et al*. (unpublished)
AvrPto1	Elicited cell death in tobacco	Greenberg *et al*. (2003)
AvrRpm1	Suppression of PTI, elicited cell death in tobacco	Greenberg *et al*. (2003)
HopA1	unknown	Hue and Hutcheson (1993); Deng *et al*. (2003)
HopA2	HR	Charity *et al*. (2003)
HopB1	HR	Charity *et al*. (2003)
HopH1	unknown	Greenberg *et al*. (2003)
HopI1	Downregulates host salicylic acid	Greenberg *et al*. (2003)
HopM1	Suppression of PTI, elicited cell death in tobacco	Lindeberg *et al*. (2005)
HopX1	unknown	Charity *et al*. (2003)
HopZ1	unknown	Sundin *et al*. (2004); Ma *et al*. (2006)
HopZ2	unknown	Ma *et al*. (2006)
HopZ3	Suppression of ETI, elicited cell death in tobacco	Ma *et al*. (2006)
HopZ3'	unknown	Charity *et al*. (2003)
HopAA1	Reduced expression of non-host resistance genes	Lindeberg *et al*. (2005)
HopAB1	unknown	Lindeberg *et al*. (2005)
HopAE1	Elicited cell death in tobacco	Greenberg *et al*. (2003)
HopAF1	Reduced expression of non-host resistance genes	Lindeberg *et al*. (2005)
HopAG1	unknown	Greenberg *et al*. (2003)
HopAH1	unknown	Greenberg *et al*. (2003)
HopAI1'	Suppression of PTI, reduced expression of plant receptor-like kinases and non-host resistance genes	Greenberg *et al*. (2003)
HopAX1	Virulence in compatible host	Losada *et al*. (2004)
HopAY1	unknown	Zumaquera *et al*. (2010)
HopAZ1	unknown	Sohn *et al*. (2012)

Hop database regularly updated and available at the *Pseudomonas*-Plant interaction website: www.pseudomonas-syringae.org

AvrXa27, PthXo1, PthXo6/7; *Pantoea agglomerans* pvs. *beta* and *gypsophilae* HsvB and HsvG), phosphothreonine lyases targeting host kinases (*P. s*. pv. *tomato* HopAl1) and mono-ADP-ribosyltransferases affecting host RNA-binding proteins (P. s. pv. tomato HopU1).

1.2.7.5. Ice nucleation activity

P. *syringae* was reported to nucleate ice by Maki *et al*. in 1974, the first instance of biological ice nuclei. The ability of Pss to nucleate ice crystals has been discussed previously in the context of atmospheric dissemination, but ice nucleation activity (INA) also has a role of some importance in pathogenesis on plants. At normal atmospheric pressure, pure water can undergo deep supercooling to its crystal homogeneous nucleation temperature of -42°C before a change of state (freezing) occurs (Debenedetti and Stanley, 2003). This is due to the absence of extrinsic nuclei used to initiate ice crystal lattice formation at warmer temperatures (Vali, 1995). Common impurities in the environment like dust particles, minerals and organic compounds serve as nuclei and allow water to freeze at temperatures near 0°C (Vali, 1995). Ice nucleating bacteria interfere with the ability of small amounts of water to undergo minimal supercooling on plant surfaces, and can raise the temperature for ice formation from -10°C to -1.5°C, a high threshold which is actually rare in nature (Southworth *et al*., 1988). By causing ice to form at relatively warm temperatures, bacteria can augment the amount of frost damage of non-cold acclimated host tissues. The benefits of increasing the release of nutrients through host cell damage may offset the detriments of irreversible modification of the habitat (Hirano and Upper, 1995).

Other INA bacteria include *P*. *fluorescens*, *P*. *chlororaphis*, *P*. *putida*, *Erwinia herbicola*, *E*. *ananas*, *E*. *uredovora*, *Xanthomonas campestris* and *X*. *campestris* pv. *translucens*; all these bacteria can be found in the phyllosphere and most are phytopathogenic (Hirano and Upper, 1995). The relationship of frost damage and Pss diseases of woody plants has been demonstrated previously in pear blossom blight (Panagopoulos and Crosse, 1964), leaf spot of sour cherry (Sule and Seemuller, 1987), and bacterial canker of peach (Weaver, 1978; Cao *et al*., 1999), apricot (Klement *et al*., 1984), prune, almond (Cao *et al*., 1999) and willow (Nejad *et al*., 2004). Sule and Seemuller (1987) further demonstrated that freeze-thaw cycling facilitated ingress of Pss into sour cherry leaves.

Bacteria with an INA phenotype are denoted as Ice^+. Clonal populations of Ice^+ bacteria display a range in temperature and frequency of INA. They have been categorized as follows: type 1 nuclei are active between -2°C and -5°C, type 2 are active between -5°C and -7°C and type 3 are active below -7°C (Fall and Wolber, 1995). Bacterial ice nuclei are outer membrane bound proteins (Warren and Green, 1985) that aggregate in small (type 1) or large (type 3) groups (Southworth *et al*., 1988; Warren, 1995). The protein consists of non-repetitive N- and C-terminus domains flanking a highly repetitive, modular central domain (Wolber and Warren, 1989) that is required for INA (Green *et al*., 1988) and was shown to be a β-helical fold (Graether and Jia, 2001). The terminal regions are thought to be the sites of anchoring to the outer membrane (Graether and Jia, 2001). The large surface area of the protein acts as a template for the formation of ice crystals (Graether and Jia, 2001) by forming a planar array of hydrogen binding groups that mimic ice lattices (Gurian-Sherman and Lindow, 1993).

The INA protein is encoded by small genes (<4kb) with a high degree of homology between Pss (*inaZ*, *inaV*) and *E*. *herbicola* (*iceE*) (Orser *et al*., 1985). High levels of expression in Pss were induced in the laboratory by shifting growth temperature from 32°C to 14-18°C, especially during stationary phase and when coupled with limitation of nutrients (nitrogen, phosphorus, sulfur and iron, but not carbon) (Nemecek-Marshal *et al*., 1993). The ice nuclei produced were of type 1, as tested by a serial dilution freezing droplet technique at variable temperatures (Nemecek-Marshal *et al*., 1993). Interestingly, it has also been shown that Pss also has a gene for a putative antifreeze protein (Feil *et al*., 2005).

Orchards and fields of frost-sensitive crops may be sprayed with competitive bacteria to minimize the extent of frost damage due to Ice^+ bacteria by exclusion (Lindow, 1987, 1988). A strain of *P*. *syringae* was mutagenized to knock out INA (Ice^-) and field trials showed promise (Lindow, 1987; Lindow *et al*., 1988; Buttner and Amy, 1989); however, public protest against genetically modified organisms ended this line of research at that time.

1.2.7.6. Cell wall degrading enzymes

Bacteria that cause soft rots such as *Erwinia carotovora*, *P*. *fluorescens* and *P. viridiflava* produce cell wall degrading enzymes (CWDEs), such as cellulase and pectate lyase,

that dissolve the middle lamella (a cementing layer of pectin between individual plant cells) and macerate the affected tissue (Agrios, 1997). To date, there has been no report of CWDEs produced by Pss; however, an analysis of the complete genome of Pss B728a revealed a cellulase family protein, a pectate lyase and a xylanase (Feil *et al*., 2005). The ability to synthesize these enzymes would help explain how Pss effectors and toxins penetrate the cell wall to reach the plant plasma membrane. In spite of the fact that the Hrp pilus may be long enough to cross a plant cell wall, this does not explain how the wall is breached initially.

1.2.8. Environmental fitness

Of all the types of bacteria that reside in the phylloplane, *Pseudomonas* is among the most commonly isolated in culture (Beattie, 2006). Pseudomonads have been isolated at high frequencies from a single host such as olive leaves (Ercolani, 1991), but are also represented to some extent from all plant speciess surveyed (Beattie, 2006). The prevalence of these bacteria in a phylloplane environment gives an indication of their competence/longevity. Phylloplane bacteria are subjected to many environmental stresses including fluctuating levels of UV radiation, temperature and humidity, desiccating winds, and low availability of water (Beattie and Lindow, 1995). These changes occur on an hourly and daily basis, but epiphytes must also contend with seasonal changes which are more extreme. Within epiphytic pseudomonad communities, non-random and continuous turnovers of the predominating species show patterns of seasonal recurrence (Ellis *et al*., 1999). Aside from the abiotic environment, the colonized surface itself plays a role in the type and quantity of epiphytic bacteria that flourish there. Populations of *P*. *savastanoi* pv. *savastanoi* on olive leaves were found to fluctuate more significantly with tree phenology and leaf age than with season or rainfall, although the latter factors also had an impact (Ercolani, 1985). Phylloplane bacteria must also compete with each other for space and nutrients which are also sought by filamentous fungi and yeasts.

Adapted bacteria increase their survival in the environment by various means. Known mechanisms that have been discussed already include production of: CLPs whose many functions include biosurfactant activity for increased motility and antimicrobial activity for reduced niche competition; EPSs for biofilm production which enhances quorum sensing

(by increasing efficiency of bacterial communication and co-ordination of behaviour in a dense, local population) and survival during times of desiccation; IAA for assisted toxin production, suppression of host responses, increased solute leakage from host cells and stomatal closure following bacterial entry; and ice nuclei to enhance frost damage which results in nutrient leakage during times of starvation and provides entry points to sheltered sites during times of extreme temperatures.

Pss copes with UV radiation damage by repairing DNA. Mutants of *uvrA* and *phr*, a nucleotide excision repair gene and a photolyase gene, were more susceptible to UVB damage than wild type (Gunasekera and Sundin, 2006). Mutants also initiated the *recA*-mediated SOS response (the arrest of the cell cycle to allow a global response to DNA damage) more rapidly than wild type (Gunasekera and Sundin, 2006). Cells were also found to move into the apoplast of bean leaves upon being exposed to UVB (Gunasekera and Sundin, 2006). UV tolerance is a phenotype that prevails among epiphytes late in the season (Jacobs and Sundin, 2001). This is also the hottest and driest time of year when fewest pseudomonads can be recovered from the phylloplane.

Flagellar motility increases epiphytic fitness, likely because it confers the ability to move to sites of lower stressors such as UV irradiation or desiccation (Haefele and Lindow, 1987). Motility does not affect pathogenicity but may be implicated in virulence (Ichinose *et al*., 2003, Tsuyoshi *et al*., 2006). There is increased motility at cooler temperatures of 18°C vs. 30°C (Hatterman and Ries, 1989). Motility coupled with chemotaxis greatly enhances survival of bacteria as they are able to move towards sources of nutrients, sites of entry, or sites of protection (Haefele and Lindow, 1987; Beattie and Lindow, 1994). Swarming motility of Pss only occurs when biosurfactant is produced and the matric water potential is at or above -0.1kPa (Dechesne and Smets, 2012).

Coping with osmotic and matric stresses is a constant effort for phylloplane bacteria. Pss can synthesize compatible solutes to minimize water stress. These include betaine, ectoine, N-acetylglutaminylglutamine amide and trehalose (Kurz *et al*., 2010). Osmoregulatory ABC transporter systems help protect bacteria against osmotic stresses on plant surfaces (Chen and Beattie, 2007).

Although copper is an essential trace element for living organisms, excessive amounts are toxic, especially to prokaryotes. For this reason, copper formulations have been used extensively in orchards worldwide as bactericides. Unfortunately, resistance to copper has increased notably (Sundin *et al*., 1989; Vanneste *et al*., 2008). Copper-resistant Pss isolates collected from these orchards were found to carry conjugative plasmids (Cazorla *et al*., 2002; Sundin and Bender, 1993; Sundin *et al*., 1989). A plasmid-borne copper resistance operon encodes three proteins in *P.s*. pv. *tomato* (Bender and Cooksey, 1986). These proteins are translocated to the periplasm and outer membrane and bind copper to sequester it from the cytoplasm (Cha and Cooksey, 1991). Pss copper resistance genes are homologous to those of *P*. *s*. pv. *tomato* (Cazorla *et al*., 2002). Some plasmids also encoded resistance to streptomycin, an alternative bactericide for orchard use (Sundin and Bender, 1993). Widespread use of antibiotic-grade streptomycin in fruit orchards of the US and Canada has ensured the stability of this trait (McManus *et al*., 2002).

Copper and streptomycin resistance have been confirmed in BC apple and pear orchards of the Okanagan Valley where *Erwinia amylovora* isolates were found to be resistant to both bactericides (Sholberg *et al*., 2000) and in blueberry fields where *P*. *syringae* was found to be resistant to copper (MacDonald *et al*., 2002). These bacteria were not examined for the presence of conjugative plasmids. In the absence of genetic resistance, bacterial EPSs enhance tolerance of heavy metals through chelation (Kidambi *et al*., 1995) and Pss alginate is upregulated in the presence of copper (Fakhr *et al*., 1999; Kidambi *et al*., 1995).

Once inside plant tissues, bacteria must cope with new stresses encountered as host defense responses. Plants have been shown to limit water availability in the apoplast to control bacteria (Freeman and Beattie, 2009; Wright and Beattie, 2004) and water-limitation also might be part of the effect of the HR (Wright and Beattie, 2004; Beattie, 2011). Epiphytic fitness may be enhanced by pathogenicity as pathogenic populations on leaf surfaces tend to be larger, survive longer, and are better at locating and colonizing protected sites than nonpathogenic populations (Beattie and Lindow, 1994a). Survival and multiplication of pathogenic and nonpathogenic strains do not show any differences under laboratory conditions but do differ under field conditions (Beattie and Lindow, 1994b).

1.3. Bacterial blight: Blueberry-*Pseudomonas* pathology

1.3.1. Symptomology

Blight diseases are characterized by sudden onset of symptoms followed by rapid decline of affected tissues. Symptoms of bacterial blight on blueberry include brown-black stem lesions (cankers) with water-soaked, irregular margins, tip dieback (apical necrosis), and bud blight; these are illustrated in Figure 1-1. Canker size is variable and may extend only a few millimeters or cover the entire length of a stem (Figure 1-1A, C, D). Cankers can enlarge over time and may coalesce, affecting the attached buds by vascular constriction (Figure 1-1C, E). Typically, one-year old canes are affected most frequently given their relative tenderness and susceptibility to predisposing factors such as frost (Figure 1-1D). Pss is not known to kill mature blueberry bushes; however, newly planted fields may have difficulty establishing new wood (scaffold limbs) as buds are repetitively killed in consecutive years with severe outbreaks of blight. Symptoms of bacterial blight usually become apparent in early spring. There are no differences in symptom expression among blueberry cultivars.

1.3.2. General plant defenses

Plant defenses are of two kinds: physical barriers and chemical protection. Physically, waxy cuticles and trichomes prevent accumulation of moisture on plant surfaces, cell walls prevent or delay pathogen access to the cytoplasm, bundled sclerenchyma cells may arrest further spread of pathogens, and stomatal width and the size and shape of associated guard cells may inhibit pathogen entry (Agrios, 1997). Plants also contain a variety of pre-formed antimicrobial compounds. These may be exuded into the environment (e.g., catechol from onion) or retained in plant cells (e.g., phenolics, tannins, saponins, lectins, etc.). Upon recognition of a pathogen, both physical and chemical defenses are induced. Cell walls may thicken, adding cross-linked phenolics, lignin, and suberin, or callose may be deposited on the inner wall in the form of papillae to trap or prevent pathogen penetration (Agrios, 1997). New physical barriers may form, such as cork layers, abscission layers, tyloses that plug xylem vessels, and gums and resins that encapsulate and isolate the pathogen (Agrios, 1997). Rapid and transient

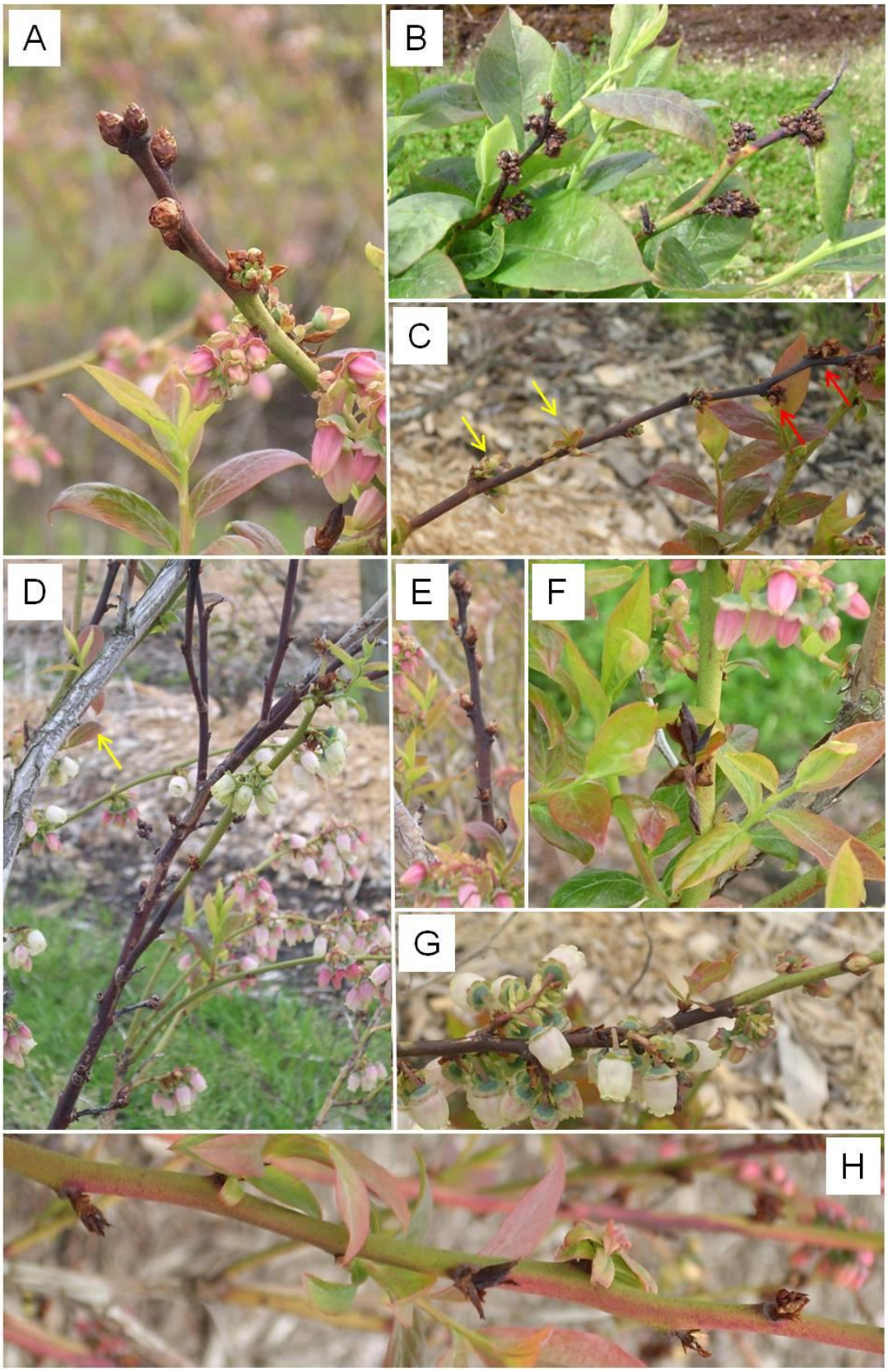
A
B
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Figure 1-1. Symptoms of bacterial blight on highbush blueberry in the field.

(A) Chocolate-brown lesions at the tip of a 1-year old cane. (B) Multiple infection sites on a single bush affecting canes and buds. Note distinct margins of necrotic regions. (C) Long lesion on a cane infecting some buds (red arrows) while other buds remain viable (yellow arrows). (D) Extensive damage to 1-year old wood while adjacent mature wood (yellow arrow) on same bush remains unaffected. (E) Long lesions extending down cane from tip and blasting of all associated buds. (F) Blast on individual bud; cane remains unaffected. (G) Floral buds on affected cane remain viable into bloom period and may succeed in setting fruit. (H) Random blasting of vegetative buds on otherwise healthy cane.

production of reactive oxygen species (ROS) can penetrate pathogen membranes (Korshunov and Imlay, 2002), cause lesions in DNA (Nunoshiba *et al*., 1999) and disrupt dehydratase function (Flint *et al*., 1993). Pathogenesis-related (PR) proteins are functionally diverse antimicrobials synthesized by plants in response to detection of pathogen elicitors or stress conditions (van Loon *et al*., 2006).

1.3.3. Specific defenses against bacteria

Plants use different defenses against biotrophic and necrotrophic pathogens and sometimes these responses are antagonistic (Collmer et al., 2009). The hypersensitive response (HR) is rapid programmed localized cell death that is effective in preventing the spread of biotrophic organisms (Hammond-Kosack and Rudd, 2008; Kliebenstein and Rowe, 2008). This defense is thought to be ineffective against Pss infection since the pathogen is quite capable of living off dead and dying tissues as a necrotroph. More refined responses occur when plant pattern recognition receptors (PRRs) recognize pathogen-associated molecular patterns (PAMPs). To date, several bacterial PAMPs have been experimentally proven to be recognized by plants. These include flagellin (Zipfel *et al*., 2004), elongation factor EF-Tu (Zipfel *et al*., 2006), lipopolysaccharide (Desaki *et al*., 2006), peptidoglycan (Erbs *et al*., 2008; Gust *et al*., 2007) and non-methylated DNA (Yakushigi *et al*., 2009). Upon recognition, PAMP-triggered immunity (PTI) responses include a signal cascade beginning with a receptor-like kinase (RLK) and resulting in transcription of PR proteins (reviewed in Bent and Mackey, 2007; Boller and Felix, 2009). Bacteria have evolved ways to silence PTI responses, namely through effectors (discussed above). Plants have responded by evolving effector-triggered immunity (ETI). This is the essence of the gene-for-gene theory of plant-pathogen interactions proposed by Flor (1947) where for each gene of host *r*esistance (*R* genes) there is a pathogen gene for *avir*ulence (*avr*) and for each gene of pathogen virulence there is a host gene for susceptibility. These interactions are illustrated in Figure 1-2.

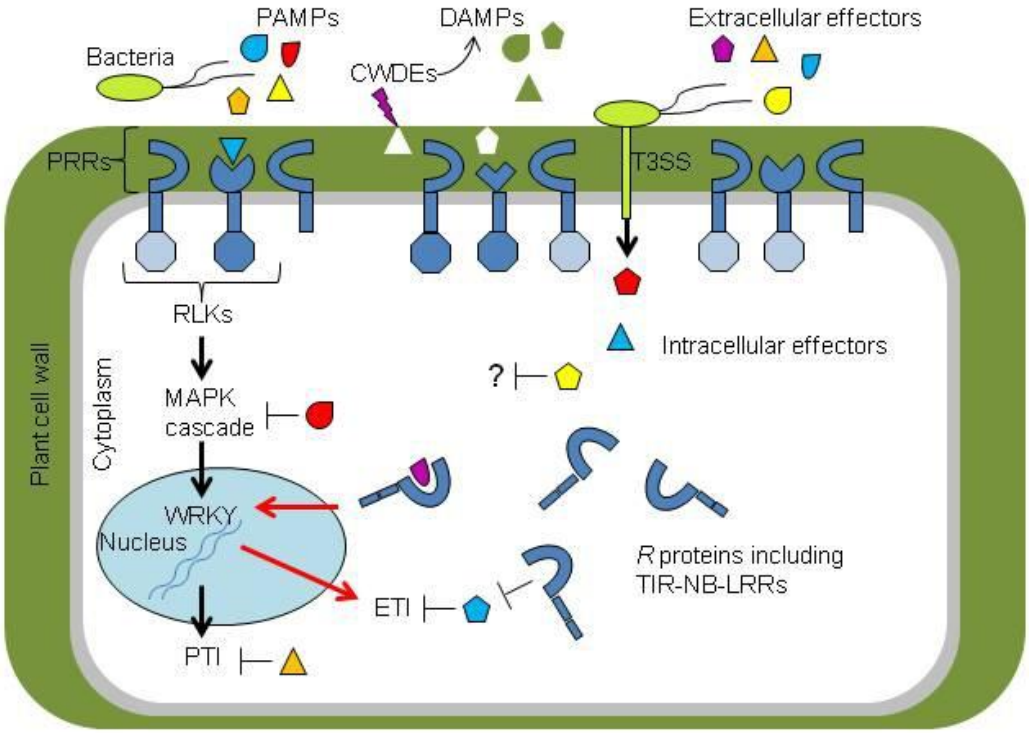

Figure 1-2. Perception and recognition of bacterial PAMPs by plant cells and subsequent blocking of defense responses by bacterial effectors.

Figure adapted from Bent and Mackey (2007) and Boller and Felix (2009). PAMPs are pathogen associated molecular patterns and include flagellin, EF-Tu and other molecules of bacterial origin. They are recognized by membrane-bound pattern recognition receptors (PRRs) and receptor-like kinases (RLKs) trigger a MAPK cascade resulting in activation of the WRKY transcription factor to initiate PAMP-triggered immunity (PTI). In parallel, damage-associated molecular patterns (DAMPs) are detected and trigger immunity when cell wall degrading enzymes (CWDEs) begin to break down cellulose and pectin. Bacteria have evolved strategies to silence PTI by injecting effector proteins which block signalling and other unknown plant defense responses. To overcome loss of basal immunity, plants have evolved effector-triggered immunity (ETI) to silence or block effectors with *R* gene proteins such as Toll- and interleukin-1-like receptor nucleotide-binding leucine-rich repeat (TIR-NB-LRRs) and proceed with defense responses. More recently evolved bacterial effectors may block ETI and plants are again adapting to resist in an on-going arms race.

1.4. History of bacterial blight on blueberry in the Fraser Valley

Published results from surveys of bacterial blight in the Fraser Valley are not available and, therefore, no records of disease severity or incidence exist. Minimal documentation based on grower submissions to the BC Ministry of Agriculture (BCMA) Plant Diagnostic laboratory (PDL) provide historical records, but cannot truly reflect the status of the disease in the area because submissions to the PDL are temporally and geographically random. Growers that recognize the disease probably would not submit samples for diagnosis except, perhaps, in years with uncommonly high blight incidence.

Samples of blueberry would normally be submitted in early spring when symptoms appear; however, damage due to cold weather would precede visible symptoms. Therefore, periods of susceptibility are thought to be in early fall (October-November) and early spring (February-May). The years 1997 and 2007 had severe bacterial blight (M. Sweeney, BC Ministry of Agriculture, pers. comm.) while in 1999 extensive damage occurred to flower buds (K. Ng, unpublished report). 2002 was a year with moderate blight following a year that had very little blight (L. MacDonald, unpublished report). The annual number of cases of bacterial blight on blueberry confirmed by the BCMA PDL is shown in Figure 1-3. The number of days with minimal temperatures at or below 0°C recorded at Vancouver International airport in the months preceding sample submission time is also shown in Figure 1-3. No clear pattern of cold weather during periods of susceptibility and the frequency of bacterial blight diagnoses is apparent from the minimal data available.

1.5. Alternative controls for bacterial blight

Because bacteria are highly mutable under selective pressure, can exchange genetic material and multiply rapidly, resistance to control products can develop and spread easily. This is especially the case with bactericides approved for use in orchard and small fruit production because there currently is only one active ingredient in all formulations, namely copper. Whether it is applied as copper oxychloride or Bordeaux mixture (copper sulfate on a slaked lime carrier), the lack of alternatives for spraying in

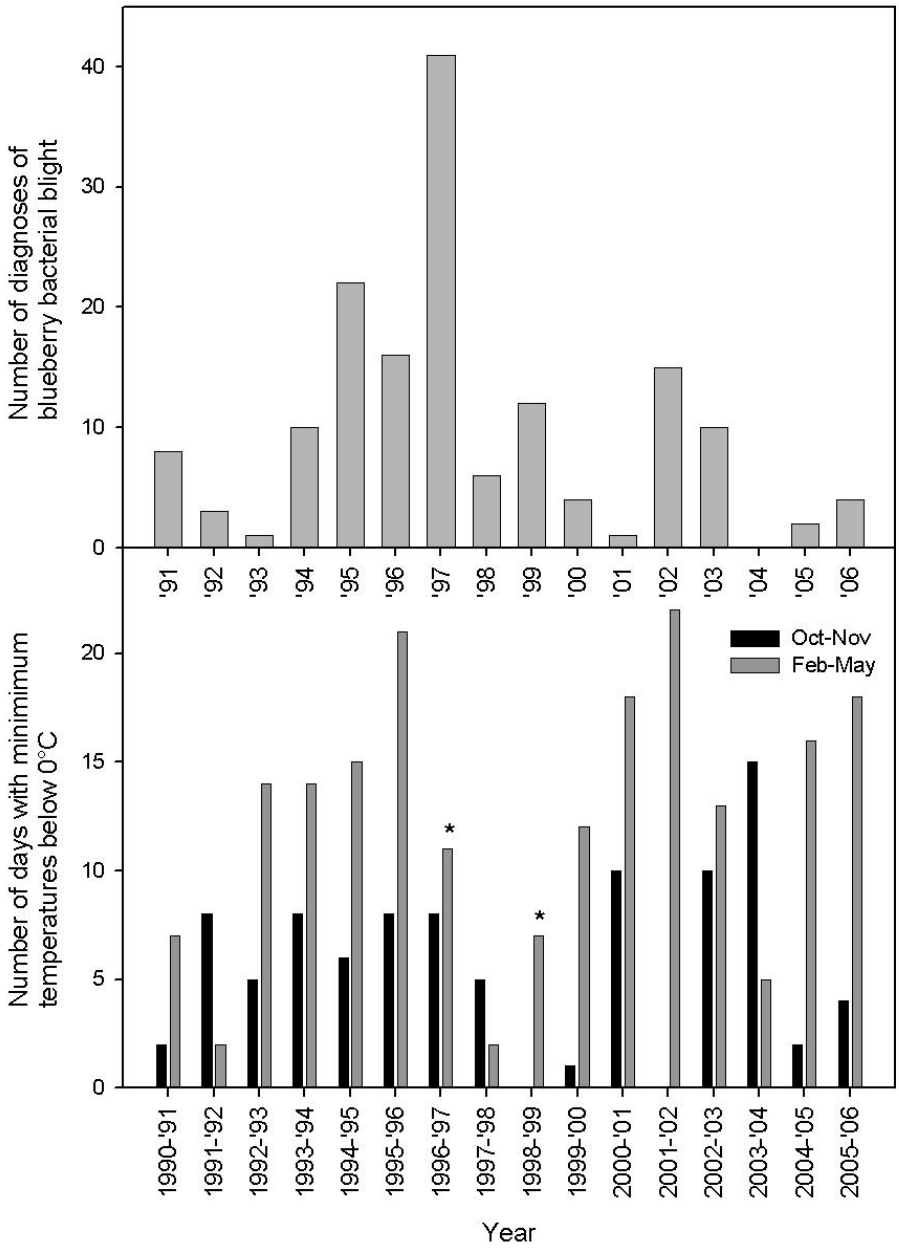

Figure 1-3. Annual submissions to BCMA PDL diagnosed as bacterial blight of blueberry and monthly days below 0°C during periods of host susceptibility(Oct.-Nov., Feb.-May).

Asterisks indicate seasons with occurrences of frost in April (a single instance each time).

rotation means that resistance is highly likely but growers have little choice. A survey of copper and streptomycin resistance in Pss isolated from woody hosts in the Willamette Valley of Oregon found that 47.8% of the total isolates and 95% of the blueberry isolates were resistant to 0.32 mM $CuSO_4 \cdot 5H_2O$ (Scheck *et al*., 1996). Blueberry isolates of Pss resistant to copper also have been detected in the Fraser Valley (MacDonald *et al*., 2002). In the 10 years since this report, copper continued to be the first choice for chemical control of bacterial blight (BCMAL, 2009). A comparison of copper-based bactericides found that only cupric hydroxide (Kocide®) combined with mancozeb (a dithiocarbamate fungicide; ManKocide) or ferric chloride had efficacy in controlling copper-resistant Pss whereas copper-sensitive strains were controlled by any copper formulation (Scheck and Pscheidt, 1998). Dithiocarbamate fungicides plus copper were demonstrated to synergistically control *P*. *s*. pv. *tomato* in laboratory and greenhouse assays; the manganese-ethylenebisdithiocarbamates such as maneb and mancozeb were particularly effective (Conlin and McCarter, 1983). Some work has been conducted on the efficacy of dithiocarbamates against clinically significant bacteria such as *Mycobacterium* (Byrne *et al*., 2007) and staphylococci (Adachi *et al*., 1997). Kocide® formulations are not registered for use in orchard or berry production in Canada.

Biocontrol agents (BCAs) such as Serenade MAX® (*Bacillus subtilis* QST 713), Bloomtime Biological™ FD (*Pantoea agglomerans* E325), Blightban C9-1 (*Pantoea agglomerans* C9-1) and BlightBan A506 (*P*. *fluorescens* A506) have been approved for used in other orchard crops by the Pest Management Regulatory Agency (PMRA) of Canada, the body of oversight for pesticide use. BCAs control phytopathogens in several ways and of relevance to the control of phytopathogenic bacteria, BCAs compete for niche occupation and also exhibit antibiosis (Pal and McSpadden-Gardener, 2006). Attenuated phytobacteria are potential BCAs but have not been commercially formulated. For example, L-forms of *P.s*. pv. *phaseolicola* (Daulagala and Allan, 2003; Waterhouse *et al*., 1994) and pv. *pisi* (Elvira-Recuenco and van Vuurde, 2003) can be induced in the laboratory and colonize plants non-pathogenically. L-forms of Pss have not been reported to date but should be inducible as well. The Ice^- strain mentioned previously could have been commercially successful under other circumstances. *Hrp* mutants of *X. campestris* pv. *vesicatoria* (Moss *et al*., 2007), *P. solanacearum* (Etchebar *et al*., 1998; Frey *et al*., 1994), and *P. s*. pv. *tomato* (Wilson *et al*., 2002) have been

demonstrated to act as BCAs of wild type phytopathogenic bacteria. Bacteriophages also have been proposed as BCAs (Jones *et al*., 2007). Phage φ6 infects *P*. *syringae* specifically (Bamford *et al*., 1987; Vidaver et al., 1973; Romantschuk *et al*., 1988; Vidaver et al., 1973) and it is possible that phages could be formulated to increase survival on target plants (Iriarte *et al*., 2007).

Cultural management strategies include other approaches to controlling disease in the field without the use of chemicals. In the case of Pss where frost damage incites a greater degree of disease, frost protection may be a viable method of minimizing losses. Citrus orchardists use sprinklers to keep fruits and leaves damp, allowing thin layers of ice to form on plant surfaces rather than within tissues. As ice crystals form, the latent heat of water is released (Perrot, 1998) and prevents frost damage. Other types of physical barriers to frost might include hydrophobic films of substances such as kaolin clay which was shown to provide protection to tomato plants down to -6°C (Wisniewski *et al*., 2000). A second strategy is to use turbine-driven windmills to churn the air and mix the cooler ground level air with warmer air above the plant canopy. Any of these practices would be necessary only during periods when blueberry plants are not cold-acclimated but there is a risk of frost. As a cautionary note, it should be stated that attempted eradication of Pss could create a void to be filled by other, more aggressive pathogens, especially given the prolonged, nonpathogenic epiphytic phase of the Pss lifestyle.

1.6. Research objectives

Pss is a well-studied organism, both as a plant-associated bacterium and as a model organism. However, less is known about Pss on woody hosts than on herbaceous plants and very little work has been done on its relationship with blueberry. The increasing popularity of blueberries has greatly extended its range of cultivation into South America, Asia, Australia and Europe. Given the sporadic nature of bacterial blight plus the ubiquity of Pss, a better understanding of the epidemiology of this disease could be used in disease forecasting and developing predictive models of outbreaks.

The purpose of this research was to (i) survey for the presence of Pss on blighted blueberry tissues in the Fraser Valley; (ii) characterize the isolates regarding their pathogenicity and other characteristics; and (iii) conduct inoculated field trials with selected pathogenic isolates to explore the survival and site of overwintering of Pss on highbush blueberry plants and whether population size influences disease development.

2. Pathogenicity and molecular characterization of *Pseudomonas syringae* strains originating from blueberry plants

2.1. Introduction

Blueberries are grown world-wide as a valuable commodity that provides many benefits to human health, including high vitamin C, antioxidant and anthocyanin levels (Connor *et al*, 2002; Kalt *et al*, 1999). Breeding of highbush blueberry plants has resulted in cultivars with desirable agronomic traits, such as enhanced yield, fruit size, soluble sugars, days to maturity and stem architecture. Despite these successes, enhanced resistance to diseases caused by fungi and bacteria has lagged considerably behind (Caruso and Ramsdell, 1995; Prodorutti *et al*., 2007).

Bacterial blight, caused by *Pseudomonas syringae*, is a sporadic but important disease in blueberry plantings in many areas of production, such as western Oregon and Washington state (Canfield *et al*., 1986; Caruso and Ramsdell, 1995; Gough, 1994; Pscheidt and Ocamb, 2008), Chile (Guerrero and Lobos, 1989), Australia (Wilson and Sampson, 1984) and the Fraser Valley of British Columbia. The pathogen has been identified as *P. syringae* pathovar *syringae* (Pss) using biochemical and molecular methods (Scheck and Pscheidt, 1998). Previous observations suggest that bacterial blight is more severe after periods of extreme weather when tissues are vulnerable i.e., prior to onset of dormancy in fall and after dormancy breaks in spring. It has been suggested that winter damage is a contributing factor to outbreaks of bacterial blight on many host plants (Buttner and Amy, 1989; Cambours *et al.,* 2005; Gross *et al*., 1984; Gross *et al*., 1988; Lindow *et al*., 1982a, Lindow *et al*., 1982b; Mo *et al*., 1995; Nejad *et al*., 2004; Kennelly *et al*., 2007; Sobiczewski and Jones, 1992; Sule and Seemuller, 1987; Weaver, 1978). Winter or frost damage may be enhanced by the presence of microbes capable of ice nucleation activity; numerous strains of ice-nucleating *P.*

syringae have been detected in pome and stone fruit orchards of Oregon and Washington (Gross et al., 1984). Blueberry plantings situated at lower elevations appear to be more susceptible to frost injury, which may damage tender stems and buds, thereby providing entry points for bacteria into plant tissues. *P. syringae* is a common plant epiphyte with a wide host range (Hirano and Upper, 2000) but can also survive internally within the apoplast (Rico *et al*., 2009; Rico and Preston, 2008). As a pathogen, Pss has a repertoire of virulence factors, including production of phytotoxins (Carpaneto *et al*., 2002; Hutchison and Gross, 1997; Mo and Gross, 1991a; Xu and Gross, 1988;), exopolysaccharides (Denny, 1995; Penaloza-Vazquez *et al*., 2004; Yu *et al*., 1999;), plant cell wall degrading enzymes (CWDEs) (Feil *et al*., 2005; Lindeberg *et al*., 2008), enzymes to detoxify host defensive compounds (Feil *et al*., 2005; Klotz and Hutcheson, 1992;), and effectors (virulence proteins) translocated by a type three secretion system (T3SS) (Collmer *et al*., 2000; Fu *et al*., 2006).

There is currently little information available on the epiphytic nature of *P. syringae* populations on blueberry plants and their relationship to disease development. In addition, the interactions of pathogenic and saprophytic populations of *Pseudomonas* species on survival have not been determined. Quantification and detection of strains able to cause disease could be valuable in predicting when disease may occur under suitable environmental conditions.

The objectives of this study were to: (i) survey the *Pseudomonas* populations associated with bacterial blight symptoms in blueberry fields in the Fraser Valley of BC; (ii) conduct pathogenicity tests under laboratory conditions with select strains; (iii) investigate characteristics that contribute to fitness of Pss isolates.

2.2. Materials and methods

2.2.1. Bacterial isolation and identification

Six commercial blueberry fields located in the Fraser Valley of BC (Abbotsford, Cloverdale and Delta) with a previous incidence of bacterial blight were sampled several times during February – May of 2008 and 2009. Blueberry twigs and buds with symptoms resembling bacterial blight (blighted blossoms and twigs and tip dieback)

were collected and brought back to the laboratory. Each sample consisted of 8-10 symptomatic twigs per field. Tissues were cut thinly with a scalpel and placed in a 2 mL Eppendorf tube containing 1 mL sterile pre-enrichment buffer (3 mM KH_2PO_4, 6 mM K_2HPO_4, 1% peptone, 20% glycerol). A single 6.35 mm chrome steel bead (Biospec Products, Bartlesville, OK) was added and the tissue was homogenized for 1 min using a MiniBeadBeater-8 (Biospec Products). The resulting macerate was streaked out on Pss-selective KBC medium (Mohan and Schaad, 1987) and incubated at room temperature (21±2°C) until bacterial colonies were visible (3-5 days). Colonies with differing morphologies were selected to represent collections from each field, sampling date, and tissue type and were re-streaked for single colony purification. The resulting bacterial growth was harvested for preservation at -80°C as cell suspensions in pre-enrichment buffer. Isolates with different colony morphologies were selected for identification to species and pathovar level. Methods used included the *l*evan production, *o*xidase, and *a*rginine dihydrolase tests of the LOPAT method (Lelliot 1966), Gram staining, motility, Biolog GN2 MicroPlate® carbon source utilization assay (Biolog Inc., Hayward, CA) and detection of the syringomycin synthetase *syrB* gene cluster (Sorensen *et al*., 1998) through PCR and DNA sequencing. This gene cluster has been used as a diagnostic tool for distinguishing Pss from other *Pseudomonas* species and pathovars (Braun-Kiewnick and Sands, 2001).

Bacterial isolates were also obtained from different ornamental hosts following the same isolation protocol. These hosts included horticultural species such as *Syringa* (common lilac), *Magnolia*, *Populus* and *Prunus* displaying bacterial blight symptoms similar to those seen on blueberry. Bacteria were isolated from other woody perennials (including *Buddleja, Forsythia* and *Hibiscus*) for comparison with blueberry isolates and to survey presence of Pss on other known hosts within the Fraser Valley.

2.2.2. Isolate characterization

2.2.2.1. Biochemical tests

Levan production is induced by high sucrose content in media such as NSA (5% w/v sucrose). Isolates were streaked on NSA and incubated at RT in the dark for 7 days. Plates were examined at 3 and 7 days for a domed mucoid appearance attributable to levan production. Type cultures of Pss, *P. aeruginosa* (levan-negative) and *Erwinia*

amylovora (levan-positive) were plated on NSA for comparison. One plate was used for each tested isolate. The experiment was conducted once.

The Kovacs test for cytochrome c oxidase was performed on freshly cultured isolates grown on KB. Small amounts of bacteria were scraped from plates with a sterile glass rod and rubbed onto sterile squares of Whatman No. 1 filter paper that had been previously soaked in a 0.1% aqueous solution of *N*, *N*, *N'*, *N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (Sigma), dried, and stored for a short period (< 3 days) in a sealed sterile tube in the dark. Production of a blue colour within 10 s at RT after applying bacteria was considered a positive oxidase reaction. *P. aeruginosa* was used as the oxidase-positive control and *E. amylovora* as the negative control. The test was repeated only for isolates that produced ambiguous results (faint or delayed colour change).

Arginine dihydrolase activity was assayed by stabbing freshly cultured bacteria into screw-cap tubes of Thornley's medium 2A (1 g/L peptone, 5 g/L NaCl, 0.3 g/L K_2HPO_4, 3 g/L agar, 1 mg/L phenol red, 10 g/L arginine-HCl, pH 7.2) and covered with 1 mL sterile mineral oil to induce anaerobic conditions within the medium. A colour shift from pale pink to red within 4 days of incubation at RT indicated a positive reaction due alkalinization by NH_3, a byproduct of arginine metabolism by facultative anaerobes. *P. aeruginosa* was used as the arginine-positive control and *E. amylovora* as the negative control. The test was repeated only for isolates that produced ambiguous results (small degree of colour shift).

2.2.2.2. Biolog®

48-h old cultures grown on KB were visually checked for purity (uniform colonies), streaked onto BUG agar (Biolog Inc., Hayward CA) and grown overnight at 30 °C. Resulting growth on BUG was transferred with a sterile cotton swab to 20 x 150 mm tubes containing 20 mL sterile Biolog inoculating fluid (0.4% NaCl, 0.03% Pluronic F-68, 0.02% gellan gum in ddH_2O) and gently vortexed. Homogeneous suspensions in pre-blanked tubes were adjusted to 52% transmittance as measured by a turbidometer (Biolog Inc.) calibrated with a turbidity standard tube (Biolog Inc.).

A volume of 100 µL of inoculating fluid was transferred to each well of the 96-well Biolog GN2 MicroPlates® using a multi-channel pipettor. Microplates were sealed in plastic bags and incubated at 30 °C for 16 h, scanned by a MicroStation plate reader and interpreted with MicroLog3 software (database version 4.20.05, Biolog Inc.). Microplates were re-read at 20 h to check for changes in probability and/or similarity scores. One microplate was used for each tested isolate.

2.2.2.3. DNA extraction and PCR analysis

For PCR, genomic DNA was extracted using the mini-prep method of Ausubel (1989). Briefly, cultures were grown in 10 mL LB overnight at RT and 100 rpm on a rotary shaker. 1 mL samples of the cultures were pelleted by microcentrifugation at 10000 rpm for 1 min (IEC Micromax, 851 rotor, Needham Heights, MA). Cells were resuspended in TE and incubated with 0.5% SDS and 10 µg/µL proteinase K to lyse cells and digest proteins. Polysaccharides and residual proteins were extracted with 10% CTAB/0.7 M NaCl, emulsified and precipitated by a chloroform-isoamyl alcohol extraction and centrifugation. Nucleic acids were purified further by phenol-chloroform-isoamyl alcohol extraction and centrifugation, precipitated with isopropanol, washed with 70% ethanol, dried briefly and resuspended in TE.

Aliquots of 50-100 ng extracted DNA were used for PCR reactions but occasionally direct colony PCR was performed for expediency. 25 µL PCR reaction volumes consisted of 1.25 U EconoTaq® DNA polymerase, 1 x buffer (Lucigen, Middleton WI), 1 µM of each primer and 100 µM of each dNTP. PCR reactions were performed in an MJ Mini thermal cycler with heated lid (100 °C, BioRad) programmed for 94°C 4 min, followed by 25 cycles of 30 s denaturing at 94°C, 30 s primer annealing at 60°C, 1 min extension at 72°C, and a final extension of 72°C for 10 min. The O'GeneRuler™ 10kb DNA ladder (Fermentas)and PCR products mixed with 6x loading dye were loaded onto 0.8% agarose gels containing 0.5 µg/mL ethidium bromide and separated by electrophoresis at 80-90V for 40 min in 0.5x TBE. Resulting bands were visualized under UV light in a Gel Doc (BioRad, Hercules, CA). All primer sequences are listed in Table 2-1.

Type cultures of Pss and other phytopathogenic bacteria such as *P. syringae* pv. *tomato* and *Erwinia amylovora* were included for comparison in all tests.

2.2.2.4. DNA sequencing

To confirm that the amplicon produced by the *syrB* primer pair (B1/B2) during PCR did positively identify isolates as Pss, the band was excised using the QIAgen gel excision kit (QIAgen Inc.) after electrophoresis. The purified DNA was then inserted into the pGEM®-T Easy vector system (Promega, Madison, WI) following the manufacturer's ligation protocol and cloned in TOPO competent cells (Invitrogen) grown overnight in LB+Amp (100 µg/mL ampicillin) at 37°C and 100 rpm on an orbital shaker. Resulting suspension cultures were plated on LB+Amp with X-gal (80 µg/mL) and IPTG (0.5 mM) and transformed white colonies were selected after growth overnight at 37°C. Transformed colonies were resuspended in LB+Amp, grown overnight as described, and then plasmids were extracted using the Wizard Plus SV miniprep kit (Promega, Madison, WI) following the manufacturer's protocol. Size of plasmid inserts was checked by PCR amplification using T7/SP6 primers prior to sequencing (Macrogen, Korea). Sequences were compared to GenBank accessions with BLAST (NCBI, U.S National Library of Medicine, Bethesda, MD). The identity of a *syrB*-positive strain as Pss was confirmed by further PCR amplification, cloning and sequencing of the ITS and 16S rDNA loci with universal primers.

2.2.3. Generation of a rifampicin-resistant mutant

Resistance to antibiotics such as rifampicin has been used to mark bacterial strains for studying phylloplane interactions between *P. syringae* and host plants in the field (Bedford *et al*., 1984; Cintas *et al*., 2006; Endert and Ritchie 1984a; Malvick and Moore, 1988a; Sundin *et al*., 1988). Pss isolates previously found to be pathogenic to lilac (see Chapter 2) were chosen for generation of rifampicin-resistant mutants. Mutants arose spontaneously on KBC medium amended with 50 µg/mL rifampicin (Sigma) and colonies with fastest growth, as judged by colony diameter, were selected as candidate mutants. These colonies were re-streaked on KBC amended with rifampicin (KBC+R) and growth was confirmed to be Pss by PCR amplification of the *syrB* gene as described previously (see Chapter 2). Mutant strains were harvested and stored at -80°C until they could be re-screened for pathogenicity on lilac and on blueberry leaf discs as described previously in chapter 2. One mutant was selected for future work based on the least change in virulence and growth rate.

Table 2-1. Primers used for PCR amplification in this study.

Primer	Region of amplification	Sequence 5'—3'	Tm (°C)	Product size (bp)	Reference
B1	*syrB* gene cluster	CTTTCCGTGGTCTTGCTGAGG	55.5	756	Sorensen et al. (1989)
B2		TCGATTTTGCCGTGATGAGTC	55.5		
27f	16S rDNA	AGAGTTTGATCMTGGCTCAG	56.2	1350	Lane (1991)
1525r		AAGGAGGTGWTCCARCC	55.4		
D21	bacterial ITS	AGCCGTAGGGGAACCTGCGG	69.7	530	Manceau and Horvais (1997)
D22		TGACTGCCAAGGCATCCACC	66.8		
syrB2211	hybridization probe	CCATTGACCGAAAAGGAAAA	51.1	222	This thesis
syrB2433		CATAGTTGGCGATGTTGGTG	54.1		
gyrB-F	DNA gyrase subunit B	MGGCGGYAAGTTCGATGACAAYTC	59.9	690	Sawada et al. (1999)
gyrB-R		TRATBKCAGTCARACCTTCRCGSGC	62.6		
actF	blueberry actin	TCAAGAGCCACGTATGCAAG	55.2	105	Miles et al. (2011)
actR		TCGCCTCATGAAGATCCTTAC	54.2		
ITS1	fungal ITS	TCCGTAGGTGAACCTGCGG	57.0	≈700[a]	White et al. (1990)
ITS4		TCCTCCGCTTATTGATATGC	53.0		

[a] Amplicon size is variable among fungal genera.

2.2.4. Pathogenicity tests

2.2.4.1. Screening on lilac

Micro-propagated plants have been used to study interactions of *Pseudomonas* species and their hosts (Rodriguez-Moreno *et al*., 2008; Scheck *et al*., 1998). Instead of potted plants in greenhouse or in controlled chamber settings, pathogenicity screening *in vitro* provides many advantages, such as reduced space requirements, rapid symptom development and pest-free host tissue. Lilac (*Syringa vulgaris* L.) is particularly amenable to micro-propagation. Cross-inoculation studies have demonstrated that Pss isolates from blueberry and lilac are capable of infecting either host (Caruso and Ramsdell, 1995; Scheck *et al*., 1998).

Pathogenicity of selected isolates was screened on tissue cultured lilac plantlets cv. "Sensation" (AgriForest Bio-Technologies Ltd., Kelowna, BC) grown at RT with a 16 h photoperiod in Magenta boxes (Sigma BioSciences, Mississauga, ON) containing 0.5x

MS salts (Murashige and Skoog, 1962), 30 g/L sucrose, 2 mg/L 6-benzylamino-purine, 100 mg/L myo-inositol and 0.4 mg/L thiamine (Scheck and Pscheidt, 1998). Plants were subcultured through stem cuttings every 3 months. Plantlets used for inoculation experiments were approximately 6 cm in height. Bacterial inoculum was initiated in 10 mL LB using stored, frozen cells. Cultures were grown for 48 h at RT and 100 rpm on an orbital shaker. Concentration was assessed by measuring the OD (λ = 590 nm) of a 1 mL sample and a second, smaller aliquot (100 µL) was taken for enumeration of bacterial cells by serial dilution plating.

Plantlets were inoculated by wounding a leaf axil with a sterilized #5 insect mounting pin and placing a 1 µL droplet of inoculum adjusted to 10^6 CFU/mL on the wound site. Droplets adhered to the axil by surface tension. Plantlets in Magenta boxes were incubated at room temperature for up to 14 days and were monitored regularly for symptom development. Positive symptoms included necrotic lesions and wilting due to collapse of affected stem tissues. Five plantlets were tested for each isolate and the isolate was considered pathogenic when all 5 plantlets developed symptoms. Isolates were re-tested on 5 new plantlets when results were ambiguous.

2.2.4.2. Serial dilution inoculation

The effect of inoculum concentration on disease progression in a susceptible host under a controlled environment was examined by inoculating lilac plantlets in two different ways. Plantlets were grown in Magenta boxes as described previously, with 5 plantlets per box. Inoculum of Pss9 was grown in 10 mL PDB for 24 h at RT and 100 rpm on an orbital shaker after which a 1 mL sample was taken for OD measurement (λ = 590 nm) to estimate concentration, which was confirmed by serial dilution plating on KBC. The inoculum was diluted with SDW to obtain a series of concentrations ranging from 10^9 CFU/mL to 10^1 CFU/mL.

Five plantlets for each concentration were then wound-inoculated as previously described and Magenta boxes were incubated under the same conditions as for growth. Applications of 1 µL of each bacterial suspension resulted in an estimated dose on the wound site that ranged from 10^6 to 10^3 CFU. The least concentrated dosages would effectively have had 0 CFU and acted as controls. Five plantlets were also dip-inoculated by submerging and gently swirling entire plantlets in 30 mL of inoculum for 30

s before returning them to their Magenta boxes. Inocula for dipping were prepared such that the range of total bacteria in suspension was 10^{7} to 10^{-2} CFU. Although plantlets were exposed to these numbers of bacteria, the number that actually adhered to plantlets after dipping was not estimated by immediate sacrifice of plantlets for bacterial enumeration. Doses at the lowest end of the inoculum range again acted as controls. Inocula viability and relative concentration was re-checked following treatments by plating 6 1-µL droplets of each dilution on KBC plates.

Inoculated plantlets were observed and rated for disease every 24 h. A rating scale of 0 – 4 was used, with 0 = healthy, 4 = dead and intermediate values representing different stages of symptoms for each of the two methods of inoculation (described in appended Table A5-1). The experiment was conducted three times.

2.2.4.3. Inoculation of blueberry leaf discs

Detached leaf assays have been used previously to investigate pathogenicity of *P. syringae* isolates (Bedford *et al*., 2003; Hammerschlag, 2000; Moragrega *et al*., 2003; Yessad *et al*., 1992) and leaf disc assays have also been used in pathogenicity screening of host-pathogen systems such as powdery mildew on grape and melon (Brown *et al*., 1999; Cohen, 1993; Olmstead *et al*., 2000), *P. s*. pv. *pisi* on tobacco (Atkinson *et al*., 1985), *P. s*. pv. *glycinea* on soybean (Orlandi *et al*., 1992) and Colletotrichum on blueberry (Ehlenfeldt *et al*., 2006). This method provided a feasible, high-throughput procedure for evaluating host response to Pss under laboratory conditions. Brown *et al*. (1999) found highly significant correlations between results from leaf disc assays, greenhouse tests and field evaluations for resistance of grape to powdery mildew. However, it should be noted that blueberry leaf tissue and stem tissue are physiologically different and that results from leaf assays may not accurately reflect the response of stem tissues to Pss infection.

Healthy, fully expanded blueberry leaves were collected periodically during the growing season (May-July) from the upper canopy of blueberry bushes in commercial fields. Leaves were selected from bushes of “Duke”, a susceptible cultivar to bacterial blight, and “Elliott”, a putatively field-resistant cultivar. Leaves were surface sterilized in 2% NaOCl + 0.01% Tween 20 for 1 min followed by a triple rinse in SDW. Single discs approximately 1 cm in diameter were punched from individual leaves using a sterilized

#4 cork borer positioned over the midvein. Inoculum of isolate Pss9 was grown overnight in PDB under conditions described above. Discs were immersed for 30 s in bacterial cell suspensions adjusted to 10^6 CFU/mL with SDW. Diluted PDB was used as the control. After completion of all treatments, inoculum viability and control PDB sterility were checked by pipetting 10 droplets on KBC. Adherence of bacteria to discs after the brief immersion was checked by plating 3 surface-dried discs on KBC.

Sterilized Magenta boxes containing Whatman No. 1 filter paper soaked in 2 mL SDW and overlaid with fiberglass mesh were used as humidity chambers. Six leaf discs were incubated in each chamber for 5 days at RT with indirect light for 16 h. Control plates were incubated under the same conditions as treated leaf discs. Discs were rated for disease development after 3 and 5 days incubation on a scale of 1 – 4, with 1 = healthy and 4 = dead (rating scale described in appended Table A5-1). Symptoms of disease included marginal necrosis (blackening) and necrosis of minor and major veins. Following the incubation period, discs were randomly selected for bacterial re-isolation after maceration and plating as previously described as well as for total RNA extraction.

Three independent trials were run for Duke and two trials were run for Elliott. Inoculation with Pss9R was done only in the first two trials with Duke and the first trial with Elliott. For the first trial on Duke, 150 leaf discs were used in each of the three treatments. In trials 2 and 3, 30 and 60 discs were used per treatment, respectively. For Elliott, 60 and 72 discs were used per treatment in trials 1 and 2. Duke trial 2 and Elliott trial 1 were conducted simultaneously, as were Duke trial 3 and Elliott trial 2.

2.2.5. Toxin production

2.2.5.1. *In vitro* fungal bioassay

Syringomycin has anti-microbial properties (Gross, 1991; Lavermicocca *et al*., 1997). Plate bioassays provide an indirect method for determining production of toxins such as syringomycin through inhibition of fungal growth. To determine whether strain Pss9 produces syringomycin, an *in vitro* assay using fungistasis as an indirect determinant of anti-microbial compound detection was conducted.

Production of syringomycin was induced *in vitro* by growth of cultures in PDB with 0.4% (w/v) casamino acids (Gross and DeVay, 1977). Pss9 plus syringomycin-positive and -

negative control isolates (*P. syringae* pv. *syringae* B728a and *P. viridiflava* 5B, respectively) were grown overnight in PDB + cas under conditions previously described. 1 mL of each 10 mL starter culture was added to 200 mL of PDB+cas in individual 500 mL flasks which were incubated quiescently for 6 days at RT. Samples were taken aseptically every 2 days for enumeration of bacteria by OD readings (λ = 590 nm) and serial dilution plating, which also served as check of purity. At the end of the incubation period, cultures were killed by adding equal volumes of acetone acidified with 1 mL 12 N HCl. The resulting mixture was centrifuged at 4°C and 11,600 x *g* (Sorvall RC6, SLA-1500 SuperLite rotor, Mandel Scientific) for 20 min to pellet cellular debris. The supernatant was then concentrated 10-fold in a rotary flash evaporator (Buchi) at 55°C and the pH was adjusted to 2 by addition of approximately 1 mL 5M KOH. This solution was then extracted 3 times with equal volumes of 1-butanol and extracts were pooled. An equal volume of SDW was added to the pooled extracts and the resulting azeotropic mixture was concentrated to 3 mL by rotary evaporation at 55°C.

Concentrated crude extracts were assayed *in vitro* against three different fungi isolated from blueberry tissue (*Botrytis, Sclerotinia,* and *Phomopsis*). Petri dishes containing PDA were marked on the underside in a cruciform pattern using a template. Eight droplets (10 µL each) of toxin concentrate or control concentrate were pipetted onto the marked areas and a fungal plug cut with a #3 cork borer was placed in the center. Radial growth of the fungus and/or the zone of growth inhibition between the fungus and the toxin droplet were measured with calipers after 3 days (*Botrytis* and *Sclerotinia*) or 5 days (*Phomopsis*) incubation at RT in the dark. The experiment was conducted three times. Crude extracts were also spotted onto 20 x 20 cm aluminum-backed TLC silica gel plates (60 F_{254}) in 10 µL aliquots to check for the presence of a band with an R_f value corresponding to that for Pss phytotoxin in an ethanol/25% ammonia/water (60:30:10) solvent system (Khan and Rudolph, 1997). Plates were examined under standard white and UV light for the presence of bands.

2.2.5.2. Detection of toxin production *in planta*

Bacterial toxins are generally considered to be virulence factors; however, there is evidence that some may also play a role in epiphytic fitness. To determine whether syringomycin was produced in the absence of other microbes on a host, axenic tissue

cultured lilac plantlets were wound-inoculated with Pss9 or control strains as previously described with 10^6 CFU/mL Pss9, Pss B728a, *P. viridiflava* 5B, or dilute sterile PDB. Five plantlets per Magenta box received one treatment and were incubated under standard conditions for 5 days. Individual plantlets were then rated for disease using the 0-4 scale, placed into 2 mL Eppendorf tubes, weighed, and frozen at -80°C. Plantlets were then lyophilized overnight and dried tissues were pulverized using a single glass bead (0.5 mm) per tube with the MiniBeadBeater-8 (Biospec Products, Bartlesville, OK) set to homogenize for 1 min. Total RNA extraction was performed using Concert Plant RNA reagent (Invitrogen) or TRIzol® (Invitrogen) according to the manufacturer's protocol. Extracted RNA was quantified by a NanoDrop spectrophotometer and quality was checked by loading aliquots mixed with denaturing buffer (MOPS, de-ionized formaldehyde, de-ionized formamide) , 10 x RNA loading dye, and ethidium bromide onto a denaturing agarose gel (1% agarose, 1x MOPS, 18% formaldehyde in DEPC-treated ddH_2O) run in 1x MOPS buffer.

5 ng RNA were used for each RT-PCR reaction. Superscript II reverse transcriptase (Invitrogen) and random hexamers (500 µg/mL) were used to synthesize first strand cDNA according to the manufacturer's protocol. 2 µl of cDNA were then used for each standard PCR reaction as described previously with primer pairs for *syrB*, bacterial DNA gyrase subunit B (*gyrB*), or blueberry actin (*act*; Table 2-1). Cycles were run with annealing temperatures appropriate to each primer pair. PCR products and a DNA ladder were loaded onto agarose gels as previously described. Resulting band sizes were checked for a match to the expected size for *syrB*, gyrase, or actin. Three independent trials were run for lilac plantlets. Blueberry leaf discs inoculated as previously described for pathogenicity trials were also tested for *in planta* expression of *syrB* following the same methods.

2.2.6. Statistical analyses

For all experiments, data were analysed with Statistical Analysis System v.9.2 software (SAS Institute, Cary IN). Normality of all data was examined with *proc univariate* and the appropriate parametric test or its non-parametric equivalent was selected for further analysis. The type 1 error rate was set at $\alpha = 0.05$ for all tests.

Data for the leaf disc pathogenicity assays were tested for analysis of variance by *proc npar1way* with pairwise comparisons of means using Tukey's HSD. Data could not be pooled for the three trials due to significant differences in variance as indicated by *F*-tests. Differences in disease ratings over time (3 dpi vs. 5 dpi) within treatments were analysed by *proc npar1way* with the *wilcoxon* option.

The serial dilution inoculation studies on lilac plantlets were analysed by calculating AUDPC values using the following formula:

$$AUDPC = \sum_{i=1}^{n} \left[\frac{x_{i+1} + x_i}{2}\right](t_{i+1} - t_i)$$

where x_i is the proportion of diseased tissue at time *t* and *n* is the number of observations (Vidhyasekaran, 2004). Trapezoids calculated using midpoint sums on the curve were then summed over 3-, 5-, 7-, and 14-day intervals of incubation. ANOVAs were conducted on AUDPC values at these intervals for all concentrations of inoculum in the two methods of inoculation. Data were pooled for three experiments and analysed by *proc GLM* with Tukey's HSD for pairwise comparisons of means.

Measurements of radial growth of fungi challenged with extracted toxins were analysed with *proc glm* and Tukey's HSD for pairwise comparisons. Data could not be pooled for the three trials due to significant differences in variance as indicated by *F*-tests.

2.3. Results

2.3.1. Bacterial isolation and identification

One hundred and twelve bacterial isolates were collected over two consecutive sampling seasons (Tables 2-2 and 2-3). Of these, 52 isolates were tested by Biolog which identified Pss (4 isolates) and *P. viridiflava* (18 isolates). Other identified phytopathogens included *P. syringae* pathovars *aceris*, *tagetis*, *apii*, *helianthi*, *coronafaciens*, *lachrymans*, *antirrhini*, and *aptata*. One isolate each of *P. tolaasii* and *P.*

fluorescens also were identified. Ten isolates were identified as *Pseudomonas* but not to species while three isolates could not be identified by Biolog. All tested bacteria were motile, Gram negative rods except one *Bacillus*-type isolate (wrinkled colony morphology and endospore formation) that was not fully identified (data not shown).

Isolates recovered in the first year of study were also subjected to partial LOPAT testing (Table 2-2, appended Fig. A2-1). All but one of the *P. syringae* pathovars were consistently levan-positive, oxidase-negative, and arginine dihydrolase-negative. *P.s*. pv. *tagetis*, as identified by Biolog, was levan negative. *P. viridiflava* isolates were consistently negative for oxidase and arginine tests but were either levan-positive, delayed-positive, or negative. Pss type cultures (Table 2-4) were tested for comparison with field isolates and all but one had the expected $L^+O^-A^-$ phenotype. Strain NCPPB281, originally isolated from lilac in 1950, did not produce detectable amounts of levan on NSA. Isolates recovered in the second year of study were not subjected to LOPAT testing since one of the tests (oxidase) is replicated by Biolog plates.

A comparison of field isolates from each of the two years showed that they were not substantially different in composition. Most colonies isolated on KBC medium were *Pseudomonas*, with many (47%) identified as *P. syringae* and its subspecific pathovars. High proportions of *P. viridiflava* were also isolated in each year (23.8% of isolates identified to species by Biolog in year 1 and 54.1% of isolates identified to species in year 2).

Pseudomonads recovered from blueberry plants were similar in profile when compared to those recovered from other local woody hosts (Table 2-5). Isolates positively identified by Biolog and PCR as Pss were also recovered from symptomatic magnolia and lilac trees. Other pathovars from horticultural trees and shrubs included *P.s*. pv. *apii*, *coronafaciens;* and *P. eriobotryae, P. cichorii* and *P. tolaasii* also were identified. Two isolates were identified as *Pseudomonas* but not to species. A single isolate from Forsythia was identified as *Stenotrophomonas maltophilia* (γ- Proteobacteria) which never was detected on blueberry using the isolation techniques described. Recovered pseudomonads from non-blueberry hosts were a mixture of levan-positive and -negative types but were consistently oxidase-negative and arginine dihydrolase-negative, supporting the Biolog results of the presence of a mixture of Pseudomonas species

Table 2-2. Identification and characterization of selected bacterial isolates from diseased blueberry tissues collected from commercial fields in spring, 2008.

Isolate	Biolog ID[a]	LOPAT profile[b]	*syrB* amplification[c]	Pathogenicity on lilac[d]
1	*P. syringae* pv. *aceris**	$L^+ O^- A^-$	+	-
2	*P. syringae* pv. *tagetis*	$L^- O^- A^-$	-	-
5B	*P. viridiflava* (*syringae*)	$L^+ O^- A^-$	-	+
9	*P. syringae* pv. *syringae*	$L^+ O^- A^-$	+	+
10	nt	$L^+ O^- A^-$	+	+
13	*Pseudomonas*	$L^+ O^- A^-$	+	-
17	*Pseudomonas*	$L^- O^- A^-$	-	-
19	*P. syringae* pv. *apii*	$L^+ O^- A^-$	-	-
20B	*P. viridiflava* (*syringae*)	$L^d O^- A^-$	-	-
21	*P. viridiflava* (*syringae*)	$L^d O^- A^-$	-	-
24	*P. syringae* pv. *helianthi*	$L^+ O^- A^-$	-	-
25	*P. viridiflava* (*syringae*)	$L^- O^- A^-$	-	+
27	*P. syringae* pv. *coronafaciens**	$L^+ O^- A^-$	+	+
28	nt	$L^+ O^- A^-$	+	+
29	no ID	$L^- O^- A^-$	-	-
30	*P. syringae* pv. *syringae*	$L^+ O^- A^-$	+	+
32	no ID	$L^- O^- A^-$	-	-
35	*Pseudomonas*	$L^+ O^+ A^+$	-	-
36	no ID	$L^- O^- A^-$	-	-
38	nt	$L^+ O^- A^-$	+	+
44	*P. syringae* pv. *apii*	$L^+ O^- A^-$	-	-
46	nt	$L^+ O^- A^-$	+	+
49	*P. tolaasii*	$L^- O^- A^-$	-	-
50	no ID	$L^- O^- A^-$	-	-
67	*P. viridiflava* (*syringae*)	$L^- O^- A^-$	-	-

[a] nt = not tested, no ID = no identity returned by Biolog, * = incorrect identification by Biolog as determined by PCR detection of *syrB*

[b] + = positive reaction, - = negative reaction, d = delayed reaction

[c] + = amplification, - = no amplification

[d] + = disease symptoms present, - = symptoms absent

Table 2-3. Identification and partial characterization of selected bacterial isolates from diseased blueberry tissues collected from commercial fields in spring, 2009.

Isolate	Biolog ID[a]	syrB amplification[b]
72	*P. viridiflava*(*syringae*)	-
73	*P. viridiflava*(*syringae*)	nt
74	*P. syringae* pv. *lachrymans**	+
75	*P. viridiflava*(*syringae*)	nt
76	*P. viridiflava*(*syringae*)	nt
79	*P. viridiflava*(*syringae*)	-
83	*Pseudomonas*	nt
85	*P. syringae* pv. *apii*	nt
86	*Pseudomonas*	nt
87	*Pseudomonas*	nt
88	*P. syringae* pv. *antirrhini*	nt
89	*P. syringae* pv. *antirrhini*	nt
90	*P. fluorescens* biotype A	-
91	*Pseudomonas*	nt
92	*P. syringae* pv. *aptata*	nt
93	*Pseudomonas*	nt
94	*P. viridiflava*(*syringae*)	-
96	*P. syringae* pv. *coronafaciens**	+
97	*P. viridiflava*(*syringae*)	nt
98	*P. viridiflava*(*syringae*)	nt
99	*P. viridiflava*(*syringae*)	nt
100	*P. viridiflava*(*syringae*)	nt
101	*P. syringae* pv. *syringae*	+
103	*P. syringae* pv. *tagetis*	-
104	*Pseudomonas*	nt
106	*P. syringae* pv. *syringae*	+
107	*P. viridiflava*(*syringae*)	-
108	*P. viridiflava*(*syringae*)	nt
110	*P. syringae* pv. *antirrhini*	-
111	*P. viridiflava*(*syringae*)	-
112	*Pseudomonas*	nt

[a] * = incorrect identification by Biolog as determined by PCR detection of *syrB*

[b] nt = not tested

Table 2-4. Bacterial type cultures used in comparative assays.

Strain designation	Bacterium	Source	LOPAT profile[a]	*syrB* amplification[b]	Pathogenicity on lilac[c]
P23	*P. aeruginosa*	compost	$L^- O^+ A^+$	-	-
1548	*Erwinia amylovora*	apple	$L^+ O^- A^-$	-	+
2421	*P. syringae* pv. *syringae*	cherry	$L^+ O^- A^-$	+	+
NCPPB 281	*P. syringae* pv. *syringae*	lilac	$L^- O^- A^-$	+	-
NCPPB 1900	*P. syringae* pv. *syringae*	bean	$L^+ O^- A^-$	+	nt
PDDCC 461	*P. syringae* pv. *syringae*	plum	$L^+ O^- A^-$	+	nt
Pss 5D19	*P. syringae* pv. *syringae*	tomato	$L^+ O^- A^-$	+	nt
Pss 132	*P. syringae* pv. *syringae*	tomato	$L^+ O^- A^-$	+	nt
DC89-1C	*P. syringae* pv. *syringae*	tomato	$L^+ O^- A^-$	+	nt
B728a	*P. syringae* pv. *syringae*	bean	nt	+	+
DC3000	*P. syringae* pv. *tomato*	tomato	$L^+ O^- A^-$	-	nt

[a] + = positive reaction, - = negative reaction, nt = not tested
[b] + = amplification, - = no amplification
[c] + = disease symptoms present, - = symptoms absent, nt = not tested

Table 2-5 Bacterial isolates from blighted tissues of ornamental woody hosts in the Fraser Valley

Isolate	Host genus	Biolog ID	LOPAT profile[a]
A	*Buddleia*	*Pseudomonas*	$L^- O^- A^-$
C	*Forsythia*	*P. syringae* pv. *apii*	$L^- O^- A^-$
D	*Forsythia*	*Stenotrophomonas maltophilia*	$L^d O^- A^-$
H	*Hibiscus*	*P. syringae* pv. *coronafaciens*	$L^+ O^- A^-$
K	*Prunus*	*Pseudomonas*	$L^- O^- A^-$
L	*Viburnum*	*P. syringae* pv. *eriobotryae*	$L^+ O^- A^-$
M	*Magnolia*	*P. syringae* pv. *syringae*	$L^+ O^- A^-$
O	*Syringa*	*P. syringae* pv. *syringae*	$L^+ O^- A^-$
P	*Populus*	*P. cichorii* (*syringae*)	$L^- O^- A^-$
Q	*Populus*	*P. syringae* pv. *apii*	$L^+ O^- A^-$
R	*Populus*	*P. tolaasii*	$L^- O^- A^-$

[a] + = positive reaction, - = negative reaction, d = delayed reaction

and/or pathovars. No isolates of *P. viridiflava* were detected from samples originating from horticultural species.

2.3.1.1. PCR analysis and DNA sequencing

Among the selected field isolates tested by PCR for the presence of the *syrB* gene, 13 isolates were identified as Pss, four of which had been identified as other pathovars by the Biolog test (Tables 2-2, 2-3). The *syrB* sequence from Pss9 had a 99% identity (*E* value = 0.0) to the Pss B301d syringomycin biosynthesis gene cluster sequence (GenBank accession U25130.2) when aligned using BLAST. The positive identification of Pss9 as pv. *syringae* was confirmed by amplification and sequencing of additional regions of the genome. The 16S rDNA sequence shared 99% identity (*E* values = 0.0) with both Pss strains NCCPB 3869 (16S rRNA gene sequence, GenBank accession AM399036.1) and B728a (complete genome, GenBank accession CP00075.1). The ITS sequence shared %100 identity with Pss B728a (*E* value = 2e-169).

2.3.2. Pathogenicity testing

2.3.2.1. Screening on lilac

Twenty-five isolates were tested on lilac and nine were found to be pathogenic (Table 2-2). Seven of these were confirmed as Pss but two were *P. viridiflava* isolates based on Biolog identification. One pathogenic isolate of *P. viridiflava* was levan-positive while the other was levan-negative. Two isolates confirmed as Pss by PCR did not produce disease symptoms on lilac under laboratory conditions. Disease symptoms on the plantlets were always initiated at the wound site. For Pss isolates, the injured leaf axil turned black within 1-3 days and the lesions progressed in both directions along the stem. Over the ensuing few days, the stem would often collapse at the wound site (Fig. 2-1). Leaves also began to turn necrotic with advancing lesions developing along the midvein. Eventually, the entire plantlet turned black and died. Plantlets affected by *P. viridiflava* isolates also showed symptoms at the wound site, but in contrast with Pss, discoloured areas were pale brown and appeared water-soaked. Spread of this type of lesion produced a wet, soft-rotted plantlet.

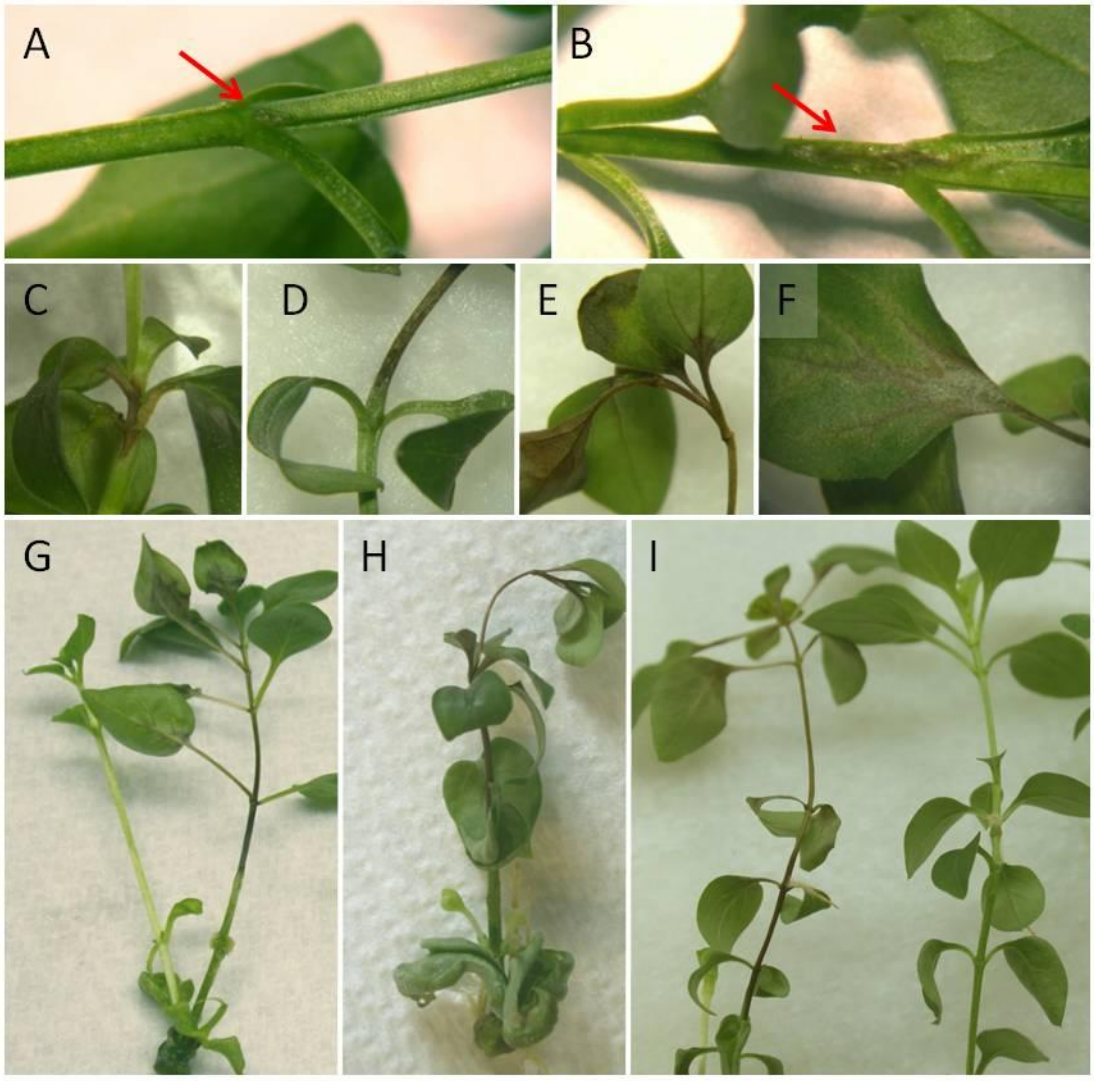

Figure 2-1. Symptoms on wounded lilac plantlets inoculated with droplets of Pss9 at 10^6 CFU/mL.

(A) Inoculation wound (red arrow) becomes necrotic within two days of incubation at room temperature. (B) Necrotic lesion (red arrow) expands and becomes sunken. (C) Lesions expand in both directions on the stem. (D) Elongated lesions maintain distinct margins. (E) Necrotic symptoms expand from stem into leaf tissues. (F) Leaf necrosis follows pattern of venation. (G) Affected plantlet with healthy pre-existing side shoot. (H) Typical wilt seen in wound-inoculated plantlets. (I) Pss9-inoculated plantlet (left) compared with mock-inoculated plantlet (right).

2.3.2.2. Serial dilution inoculation

Lilac plantlets that were wound-inoculated with Pss9 showed differences in disease progression in response to inoculum dose, resulting in a range of disease progress curves (Fig. 2-2). Generally, increasingly concentrated inocula produced earlier symptoms, which progressed more rapidly in comparison with dilute inoculum. In three trials, inoculations with 10^5 CFU showed onset of visible symptoms 1 dpi while 10^4 CFU produced visible symptoms at 2 dpi. Inoculum of 10^1 CFU did not produce any visible symptoms within the 14-day incubation period. Above the range of 10^5-10^1 CFU, inocula did not cause disease to progress at a rate significantly different from 10^5 CFU while inocula below the range also did not produce symptoms (data not shown). Much less variability was seen in disease progress with the more concentrated inocula than with the dilute inocula (Fig. 2-2). The minimum effective dose required for symptom development on wounded lilacs was estimated to be 10^2 CFU and required 6 days of incubation; however, limited disease progress occurred after this period. The minimum dose required for 100% mortality within 14 days was 10^4 CFU which produced symptom onset in 2 days.

AUDPC values calculated for four intervals of incubation showed a significant difference for 10^5 CFU compared with all other inocula at 3, 5, and 7 dpi (p=0.008, Table 2-6). By 14 dpi, disease curves initiated by 10^5 and 10^4 CFU were no longer significantly different from each other but did differ from all other treatments (p<0.001).

By its nature, the dip method of inoculation produced different disease patterns and progress due to the development of multiple foci of infection. Plantlets that received higher doses of Pss9 had multiple pale-brown water-soaked lesions that were initially apparent on the leaves and spread to the stems. By 14 dpi, many of the plantlets were severely diseased regardless of the inoculum concentration (Fig. 2-3). At lower doses of bacteria, infection most often began at the base of the plantlet where it was inserted into the growth medium. Because entire plantlets were immersed in bacterial suspensions, some bacteria came in contact with the medium after the plantlet was replaced in the Magenta box and bacterial growth on the medium then re-colonised the plantlet. Because tissue cultured plantlets are extremely vulnerable to desiccation, they could not be allowed to dry after dip treatment. Still, a range of disease progress curves was

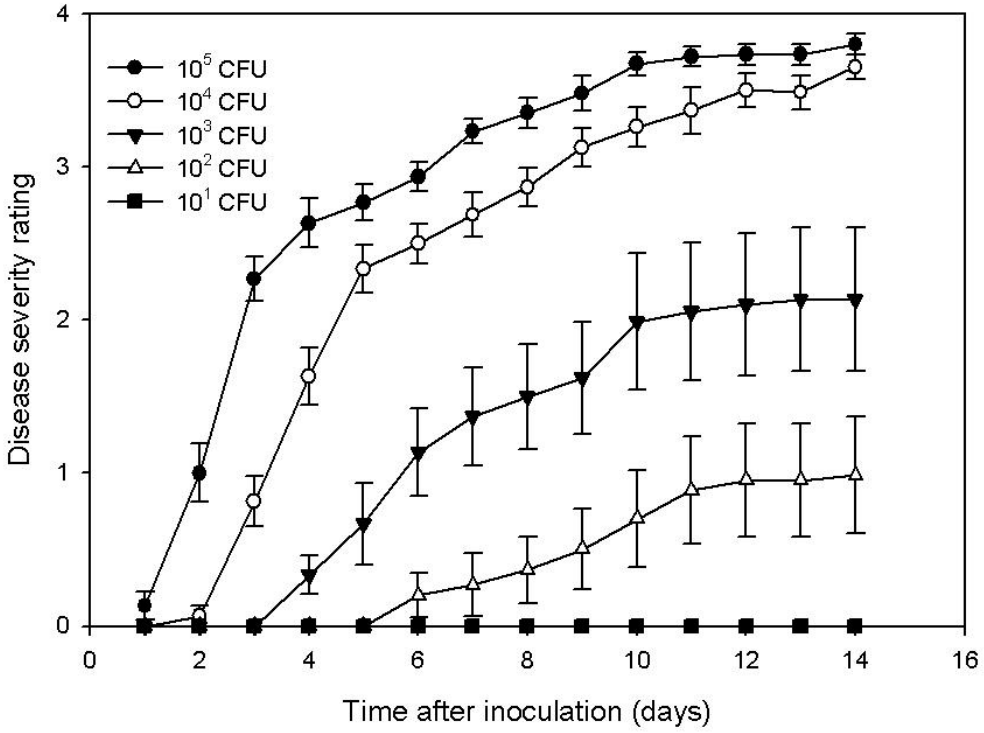

Figure 2-2. Dosage effect and disease progression in wound-inoculated lilac plantlets treated with Pss9.

Tissue-cultured plantlets were grown in Magenta jars of MS medium at RT with a 16h photoperiod. Planlets were wounded in the axil with a sterile pin and a 1 µl droplet of inoculum was applied to the wound site. Pss9 inoculum was serially diluted in SDW to produce a range of concentrations. Inoculated plantlets in jars were incubated for 14 days with daily assessments of symptom development on a 0-4 scale. Means represent the combined values for three independent trials. Error bars represent standard error of the mean (n = 15).

Table 2-6. Mean AUDPC values over 3-, 5-, 7-, and 14-day intervals for lilac plantlets inoculated with Pss9 by two different methods for a series of concentrations.

		Interval post-inoculation (days)			
Method	**CFU**	**3[ab]**	**5**	**7**	**14**
Wound	10^5	2.2 (0.3) a	7.4 (0.5) a	13.3 (0.6) a	38.5 (1.2) a
	10^4	0.5 (0.1) b	3.7 (0.3) b	8.7 (0.5) b	31.5 (2.2) a
	10^3	0.0(0) b	0.7 (0.4) c	2.8 (1.0) c	16.0 (3.7) b
	10^2	0.0 (0) b	0.0 (0) c	0.3 (0.2) cd	5.3 (1.0) c
	10^1	0.0(0) b	0.0 (0) c	0.0 (0) d	0.0 (0) c
		p=0.008	*p*=0.011	*p*<0.001	*p*<0.001
Dip	10^6	0.5 (0.2) a	2.6 (0.6) a	6.4 (1.1) a	28.4 (2.2) a
	10^5	0.1 (0.1) a	1.4 (0.8) b	3.9 (1.9) a	21.9 (5.5) a
	10^4	0.03 (0.03) a	0.6 (0.4) b	3.1 (1.2) a	21.8 (3.1) a
	10^3	0.1 (0.1) a	0.8 (0.4) b	2.6 (1.4) a	15.0 (6.7) ab
	10^2	0.0 (0) a	0.0 (0) b	0.0 (0) a	0.0 (0) b
		p=0.079	*p*=0.046	*p*=0.059	*p*=0.007

[a] Values in parentheses represent standard error of the mean.

[b] Values followed by different letters are significantly different within a column for each method of inoculation as determined by Tukey's HSD test (p values at end of columns).

produced from three trials that generally followed the pattern of more rapid disease with concentrated inocula compared to dilute inocula (Fig. 2-4). As with the wound-inoculated plantlets, the concentrated inocula produced less variability in ratings than the dilute inocula. The range of concentrations necessary to yield ratings comparable with wound-inoculation was one order of magnitude greater (Fig. 2-4). For example, 10^6 CFU were required to achieve visible symptoms at 1 dpi and the same degree of severity produced by 10^5 CFU in a wound inoculation. Correspondingly, no visible symptoms developed within 14 days following a dip in inoculum adjusted to 10^2 CFU. Some curves were not as discrete in the dip method as those for the wound method. For example, the 10^4 CFU curve fell below the 10^3 curve at 4 dpi, then crossed over the 10^5 curve at 6 dpi (Fig. 2-4).

The minimum effective dose delivered by the dipping method was 10^3 CFU, with the caveat that re-inoculation by bacterial growth on the medium augmented the dosage.

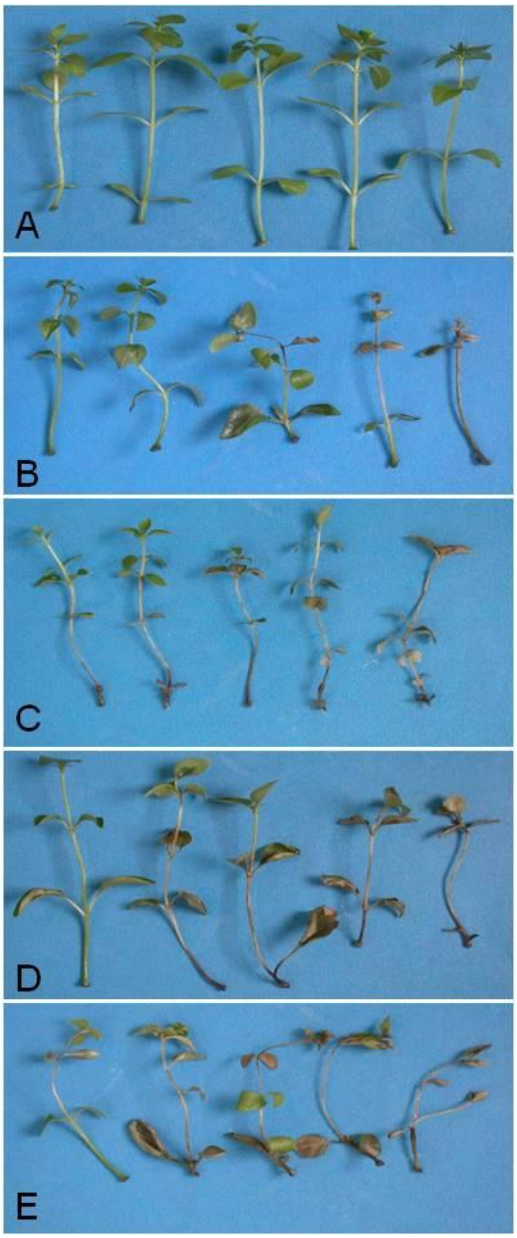

Figure 2-3. Symptoms of Pss9 on lilac plantlets14 days after application of inocula by submersion in a range of bacterial doses.

(A) 10^2 CFU produce no symptoms. (B) 10^3 CFU produce necrosis and wilting. (C) 10^4 CFU caused symptoms similar to 10^3 CFU. (D) 10^5 CFU produce advanced necrosis. (E) 10^6 CFU produce advanced necrosis and wilting.

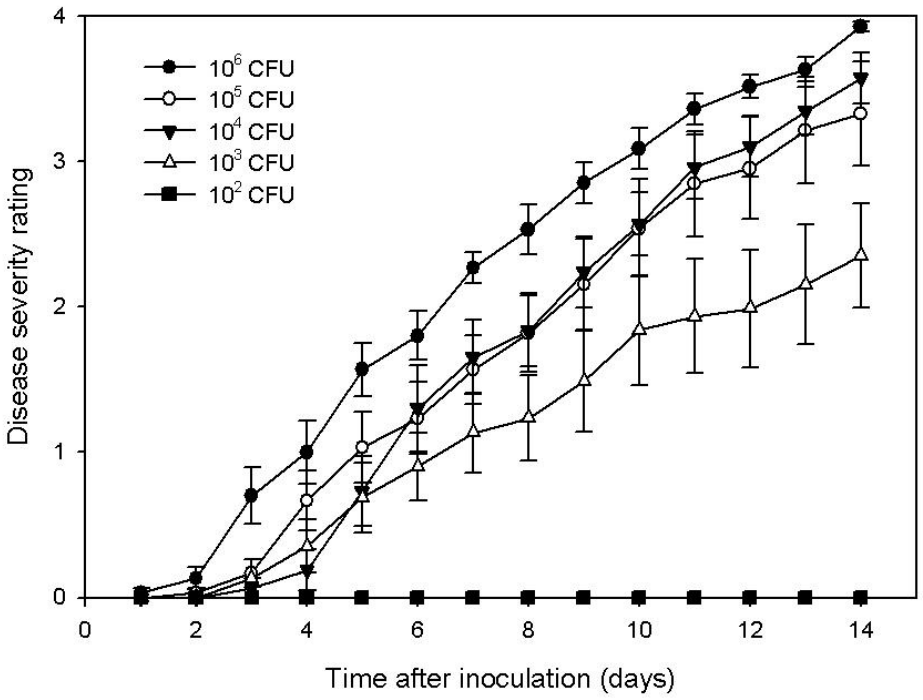

Figure 2-4. Dosage effect and disease progression in lilac plantlets submerged in Pss9 inocula.

Tissue-cultured plantlets were grown in Magenta jars of MS medium at RT with a 16h photoperiod. Plantlets were submerged in inoculum for 30 s. Pss9 inoculum was serially diluted in SDW to produce a range of concentrations. Inoculated plantlets in jars were incubated for 14 days with daily assessments of disease severity on a 0-4 scale. Means represent the combined values for three independent trials. Error bars represent standard error of the mean (n = 15).

This is supported by the fact that symptoms appeared 3 dpi while 10^3 CFU applied in a single location in the wound-inoculated plantlets required 4 days for symptom development. The dose required to cause near 100% mortality within 14 days was 10^6 CFU.

AUDPC values for dip-inoculated lilacs (Table 2-6) showed no significant differences among tested inocula at 3 dpi or 7 dpi. Plantlets treated with 10^6 CFU had a significantly different AUDPC value at 5 dpi (p=0.046) and at 14 dpi, 10^6-10^4 CFU produced significantly different AUDPC values compared to 10^2 CFU (p=0.007).

2.3.2.3. Inoculated blueberry leaf discs

Over three independent trials, Duke leaf discs inoculated with Pss9 consistently displayed symptoms of necrosis beginning at the disc margin and proceeding into major and minor veins, then into interstices. Controls and discs inoculated with Pss9R had significantly less necrosis (p<0.001) than Pss9-inoculated discs in all trials (Figs. 2-5, 2-6). In the first trial, necrotic tissue on control discs was qualitatively different, typically consisting of a minor discolouration at the disc margin that did not advance into the leaf veins. Healthy-appearing callus frequently developed at the margins of controls during this trial. However, by the time of the third trial, necrosis on control discs was similar in quality to that seen on bacterial-treated discs.

Leaf discs were rated at both 3 and 5 dpi, with significant disease progress between rating dates in both Pss9 and Pss9R inoculated (p<0.001, <0.001) and control (p=0.03) discs during the first trial and significant progress only for inoculated discs (p<0.001, <0.001) during the second trial (appended Table A4-1). In the third trial, disease progression from 3 dpi to 5 dpi was not significant for any treatment; however, at 3 dpi, more necrosis was seen on control discs than in previous trials and, conversely, less necrosis was seen on inoculated discs by 5 dpi.

No bacteria were recovered from macerated control discs at 5 dpi for any trial on either KBC or NA plates (Fig. 2-5). Lack of bacterial growth on non-selective medium indicated the absence of contaminating microbes on or in Duke leaf tissue and it was assumed that any recovered bacteria were actually re-isolations of the inocula. There were no significant differences in bacterial recovery on the different media for any treatment

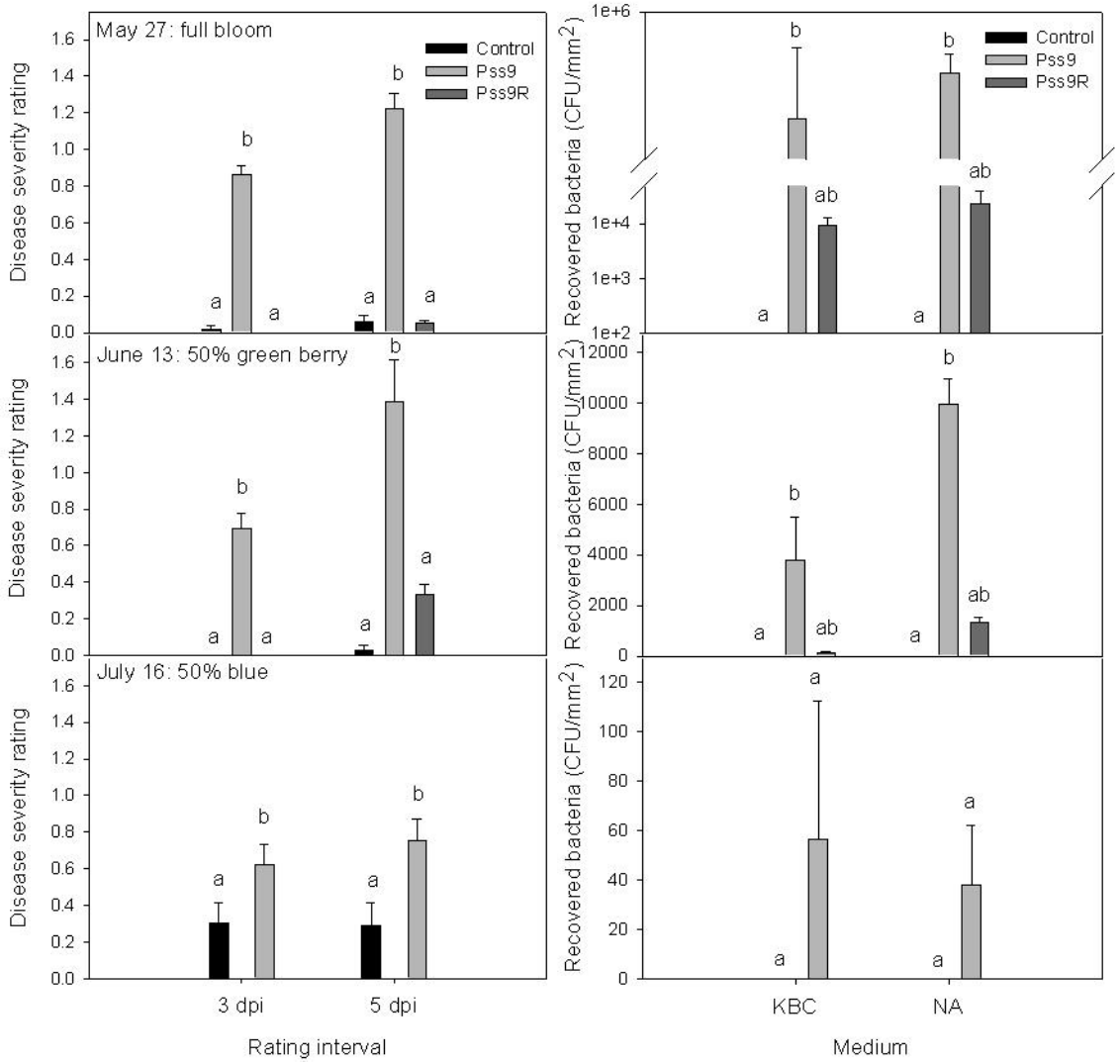

Figure 2-5. Effect of Pss inoculation at 10^6 CFU/mL on field-grown Duke leaf tissue selected at three different growth stages.

(A) Disease severity rating on a 0-5 scale and disease progression over two incubation intervals for inoculated and control leaf discs. (B) Associated bacterial populations recovered 5 dpi on Pss-selective (KBC) and general microbiological media (NA) from the discs rated in (A). Bars with the same letter are not significantly different within a rating interval or medium as determined by Tukey's HSD ($\alpha = 0.05$). Error bars represent the standard errors of the mean ($n_1 = 150$, $n_2 = 30$, $n_3 = 60$).

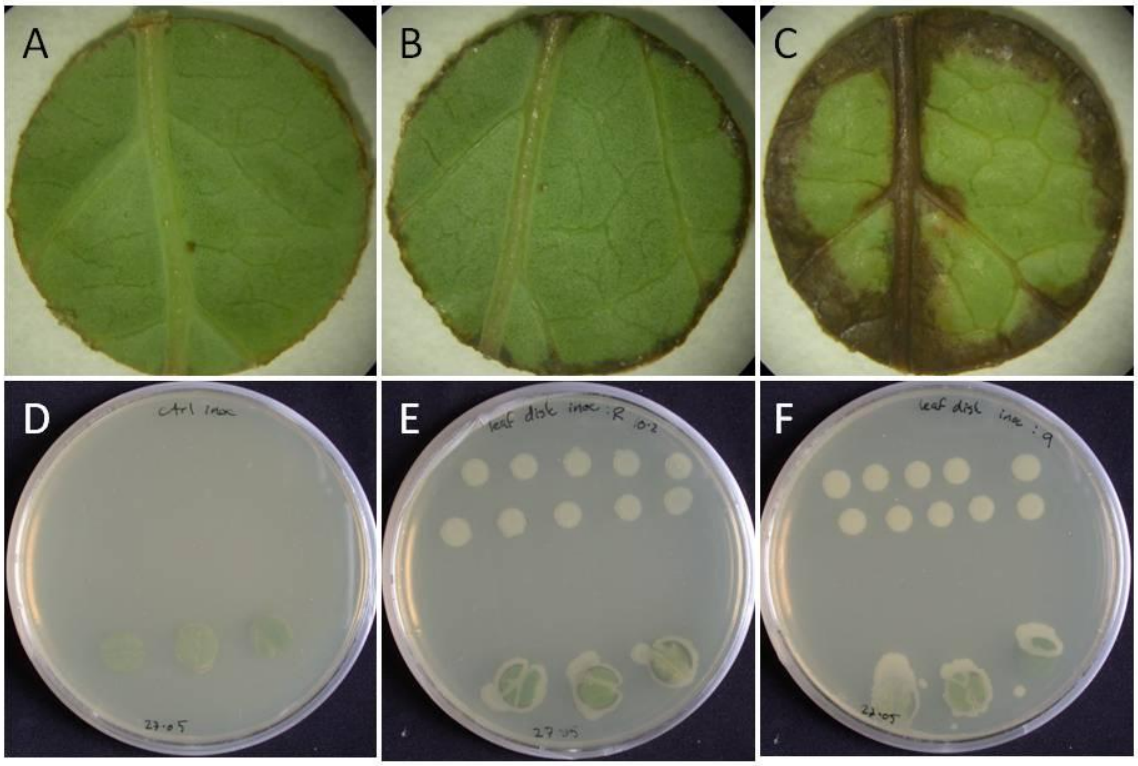

Figure 2-6. Representative Duke leaf discs at 5 dpi for trial 1 and control plates used to check inocula viability and bacterial adhesion to dipped leaf discs.

(A-C) Treated leaf discs. (D-F) Check plates; medium is KB-C. (A) Control leaf disc floated on sterile buffer for 30s. (B) Pss9R-treated disc floated on inoculum adjusted to 10^6 CFU/mL for 30s. (C) Pss9-treated disc floated on inoculum adjusted to 10^6 CFU/mL for 30s..(D) Check plate for control buffer sterility and presence of bacteria on surface-sterilised leaf discs. (E) Check plate for viability of Pss9R inoculum and adhesion of bacteria to leaf discs. (F) Check plate for viability of Pss9 inoculum and adhesion of bacteria to leaf discs.

except in trial 2 where more bacteria grew on NA for both Pss9- (p=0.033) and Pss9R-treated discs (p=0.003) (appended Table A4-1).

Numbers of recovered bacteria from Pss9-treated discs were significantly different from the control for trials 1 (p=0.004) and 2 (p=0.004) but not for trial 3 (Fig. 2-5). Pss9R recovery was never significantly different from the control.

Quantities of bacteria recovered from Pss9-inoculated discs were lower from trial 1 to trial 2 while average disease ratings and progression did not appear to differ. Differences in bacterial recovery were even greater between trials 1 and 3: a 1 x 10^3-fold decrease in recoverable bacteria was observed, but this was coupled with decreased disease severity and insignificant disease progress in the third trial. The steady decline in re-isolated bacteria occurred despite applying the same initial inoculum concentration for all trials.

Symptoms on Elliott discs were the same as Duke except that they were more pronounced. By 5 dpi, symptoms on Elliott were almost twice as severe as that seen on Duke. For two trials with Elliott, Pss9-treated discs had significantly more necrosis at 3 (p<0.001 for each trial) and 5 dpi (p<0.001 for each trial) than control discs or than Pss9R-treated discs in the single trial that included that treatment (Figs. 2-7, 2-8). Necrosis on Pss9R-treated discs did vary significantly from the control by 5 dpi (p<0.001), which was an effect not seen on Duke discs.

In the first trial, disease ratings increased significantly between 3 and 5 dpi for all Elliott discs (all p<0.001), whether inoculated or not, but only inoculated discs showed significant disease progression in the second trial (p<0.001, appended Table A4-2). Although control discs were more necrotic in the second trial than in the first, relatively large lesions developed early then failed to progress further within the time frame of the experiment. Bacteria never were recovered from the controls on either medium for either trial. The numbers of bacteria recovered from Elliott discs in each trial did not differ significantly on different media but were also very low (Fig. 2-7). For example, in trial 1 the average CFU/mm^2 of re-isolated Pss9 on KB was 5.6 x 10^1 (±5.4 x 10^1). This represented a large decline from the 10^6 CFU/ml applied.

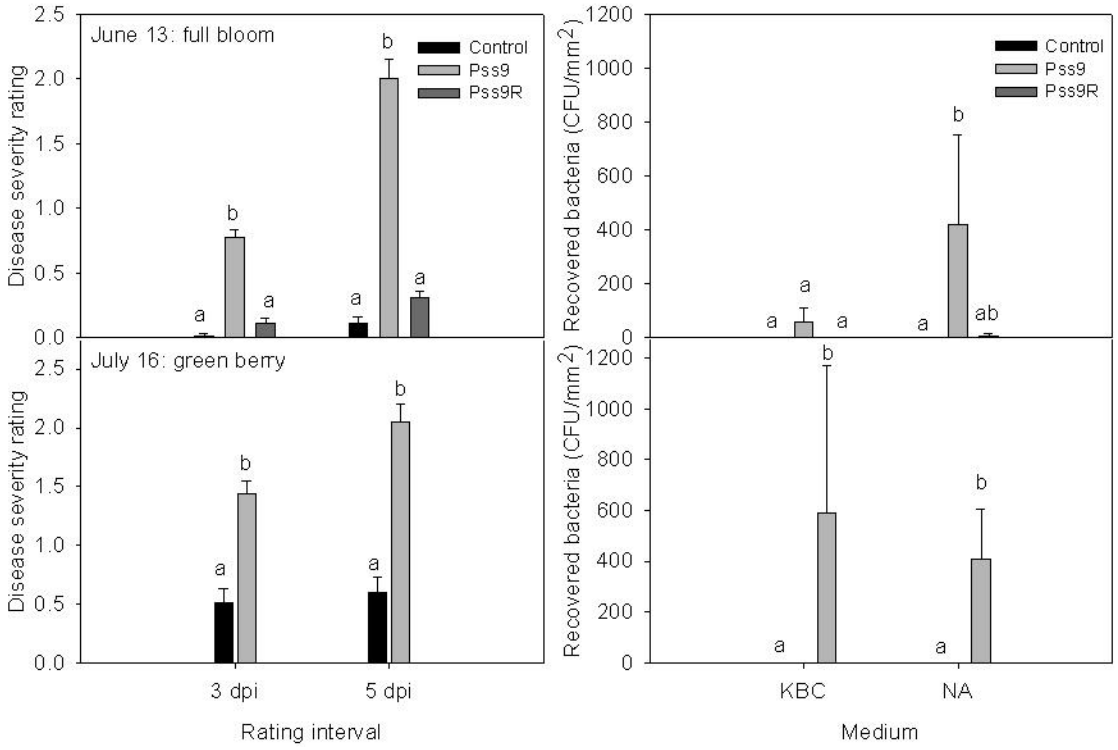

Figure 2-7. Effect of Pss inoculation at 10^6 CFU/mL on field-grown Elliott leaf tissue selected at two different growth stages.

(A) Disease severity rating on a 0-5 scale and disease progression over two incubation intervals for inoculated and control leaf discs. (B) Associated bacterial populations recovered 5 dpi on Pss-selective (KBC) and general microbiological media (NA) from the discs rated in (A). Bars with the same letter are not significantly different within a rating interval or medium as determined by Tukey's HSD ($\alpha = 0.05$). Error bars represent the standard errors of the mean ($n_1 = 60$, $n_2 = 72$).

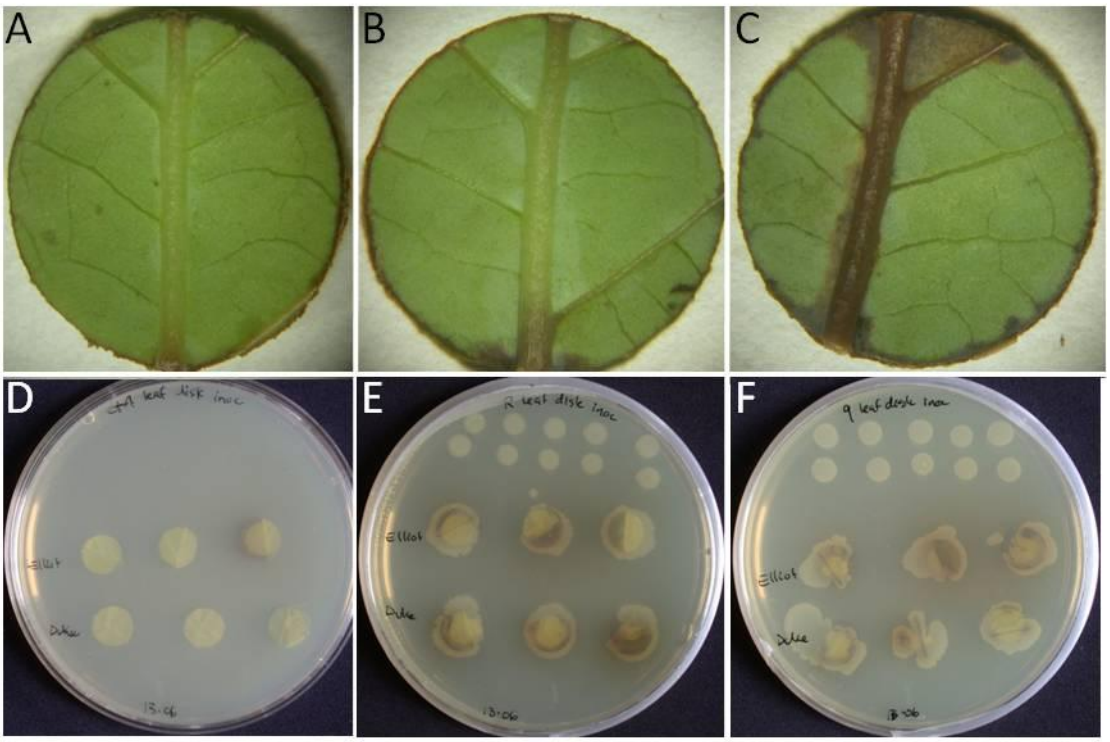

Figure 2-8. Representative Elliott leaf discs at 5 dpi for trial 1 and control plates used to check inocula and bacterial adhesion to dipped leaf discs.

(A-C) Treated leaf discs. (D-F) Check plates; medium is KB-C. (A) Control leaf disc floated on sterile buffer for 30s. (B) Pss9R-treated disc floated on inoculum adjusted to 10^6 CFU/mL for 30s. (C) Pss9-treated disc floated on inoculum adjusted to 10^6 CFU/mL for 30s. (D) Check plate for control buffer sterility and presence of bacteria on surface-sterilised leaf discs. (E) Check plate for viability of Pss9R inoculum and adhesion of bacteria to leaf discs. (F) Check plate for viability of Pss9 inoculum and adhesion of bacteria to leaf discs. Note brown discolouration of medium as phenolics leach from leaf tissues in (E) and (F).

2.3.3. Toxin production

2.3.3.1. *In vitro* toxin assays

Assays showed that both Pss9 and B728 extracts significantly inhibited radial growth of *Botrytis* (p<0.001, Fig. 2-9) and *Sclerotinia* (p<0.001, appended Figs. A1-1, A1-3) for 3 days and *Phomopsis* (p<0.001, appended Figs. A1-2, A1-3) for 5 days in comparison with extracts of PDB and 5B in each of three trials. Pss9 and B728 extracts maintained some anti-fungal activity for 7 days against *Botrytis* and *Phomopsis* but not for *Sclerotinia* (appended Fig. A1-3). All three fungal genera were isolated from blueberry stems but had different growth rates under laboratory conditions: *Sclerotinia* was fast, *Botrytis* intermediate and *Phomopsis* was slow, indicating broad-spectrum fungistasis of the extracts. Autoclaved extracts of Pss9 and B728 also were able to inhibit fungal growth significantly (p<0.001, p<0.001) except in two trials of autoclaved Pss9 extract assayed against *Phomopsis*.

Crude extracts of bacterial fermentations were run on TLC plates and UV-quenching bands that corresponded to orange-brown bands under white light were found for all treatments (Table 2-9). R_f values were not consistent over the three trials although results from trials 2 and 3 were similar or identical for bands of PDB-autoclaved, Pss9, Pss9-autoclaved and B728-autoclaved extracts. Autoclaving caused precipitates to form in all solutions and bands with smaller R_f values were seen for each extract (Table 2-9). R_f values for Pss9 and B728, the syringomycin-producing positive control isolate, were not always similar (Table 2-9) although these extracts showed the same level of fungistasis in the plate assays.

2.3.3.2. Detection of toxin *in planta*

Bands corresponding to *gyrB* were detected in lilac tissues treated with isolates 5B, B728a, and Pss9 (Fig. 2-10) when PCR was performed using cDNA template generated from RNA extracted from inoculated lilacs. Bands for *syrB* were detected also, but only for B728a and Pss9 inoculations. Both genes are single-copy on the bacterial chromosome but the *syrB* band intensity was much greater. No bands of the expected size were found for actin.

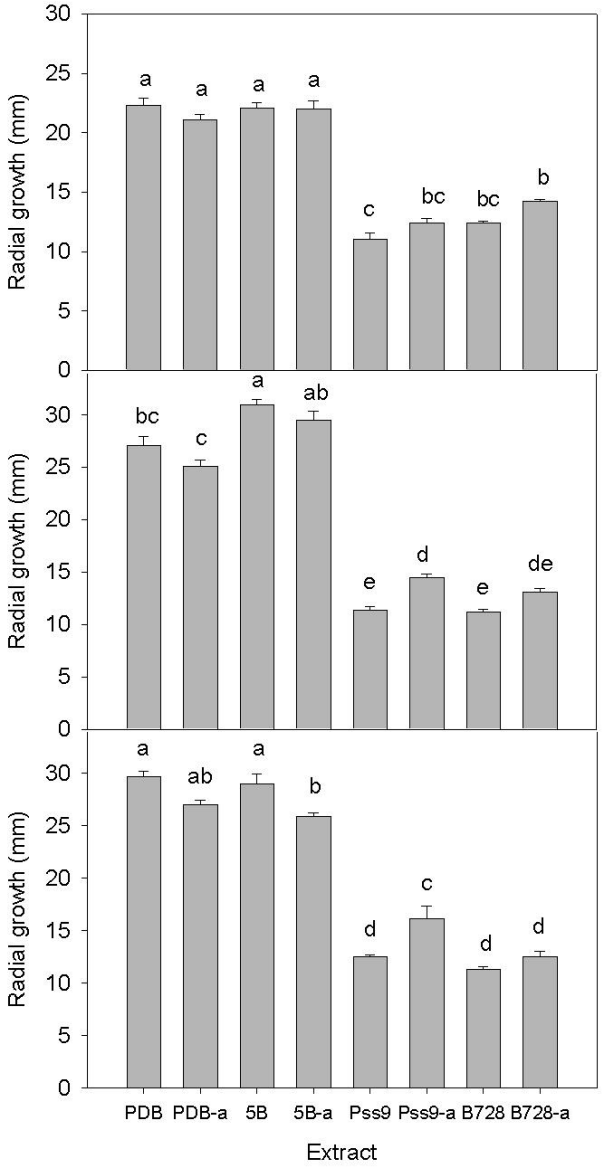

Figure 2-9. In vitro growth inhibition assays of Botrytis challenged with crude bacterial extracts and autoclaved extracts.

Top, centre, and bottom panels represent results from three independent trials. PDB = extraction of sterile potato dextrose broth, PDB-a = autoclaved extract of sterile broth, 5B = *P. viridiflava* syringomycin-negative control, 5B-a = autoclaved extract, B728 = Pss syringomycin-positive control, B728-a = autoclaved extract. Fungal plugs and 10 µL droplets of extracts were co-plated on PDA. The zone of inhibition between droplets and mycelia was measured after 3 days incubation. Bars with the same letter are not significantly different for that trial. Error bars represent the standard error of the mean.

cDNA synthesized from inoculated blueberry leaf RNA also showed bands for *syrB* when tissue was inoculated with B728a and Pss9 but not for 5B (Fig. 2-11). No bands of the expected size were detected for gyrase from blueberry tissue and faint bands were detected for actin. Since RNA quality was poor, as indicated by the denaturing gel electrophoresis and Nanodrop absorbance curves (data not shown), quantification of signal intensity could not be normalized to 23S rRNA as planned.

Table 2-9. R_f values of UV-quenching bands resulting from extracts spotted onto TLC plates.

	Trial		
Extract[a]	**1**	**2**	**3**
PDB	0.56	0.44	0.49
PDB-a	0.48	0.42	0.42
5B	0.61	0.51	0.59
5B-a	0.56	0.40	0.35
Pss9	0.68	0.55	0.54
Pss9-a	0.53	0.43	0.43
B728	0.65	0.50	0.47
B728-a	0.54	0.44	0.45

[a] Autoclaved (121 °C, 15 p.s.i., 20 minutes) extracts are denoted by –a.

2.4. Discussion

Blueberry plants in the Fraser Valley were found to contain a diverse community of pseudomonads, some of which have been reported as phytopathogens. Pseudomonads are frequently isolated from the phylloplane of many plants (Beattie, 2006) and have been reported to comprise large portions of bacterial communities on healthy leaves of olive (*P. syringae*; Ercolani, 1991), mango (*P. syringae*; de Jager *et al*., 2001) and orange trees (*P. oleovorans*, Yang *et al*., 2001) when using culture-dependent methods. Plant stems have been described as having limited bacterial communities (Beattie, 2006); however, this may not be true of perennial woody species.

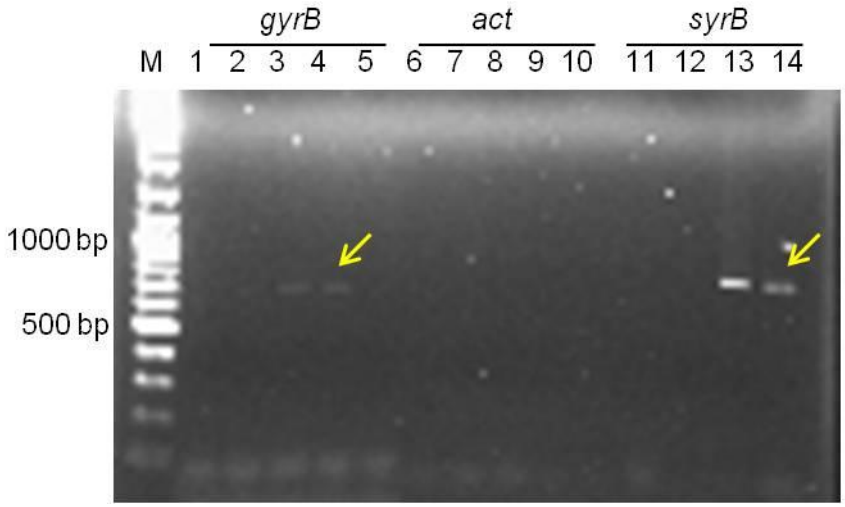

Figure 2-10. PCR detection of syrB expression in lilac plantlets inoculated with bacterial isolate 5B, Pss9, or B728a and mock-inoculated controls.

Total RNA extracted from inoculated plantlets served as a template for cDNA synthesis using random oligonucleotides as primers and reverse transcriptase. Resulting cDNA was then amplified by PCR using primer pairs for bacterial DNA gyrase (*gyrB*), plant actin (*act*) or syringomycin synthetase (*syrB*) and PCR products for each primer pair were loaded onto the gel as indicated. M is the 10 Kb O'geneRuler DNA ladder (Fermentas). Yellow arrows indicate bands of expected size for the amplicon indicated. Lanes 1, 6, and 11 are mock-inoculated controls. Lanes 2, 7and 12 are 5B syringomycin-negative controls. Lanes 3, 8 and 13 are Pss9-inoculated. Lanes 4, 9and 14 are B728a syringomycin-positive controls. Lanes 5 and 10 are PCR master mix blanks.

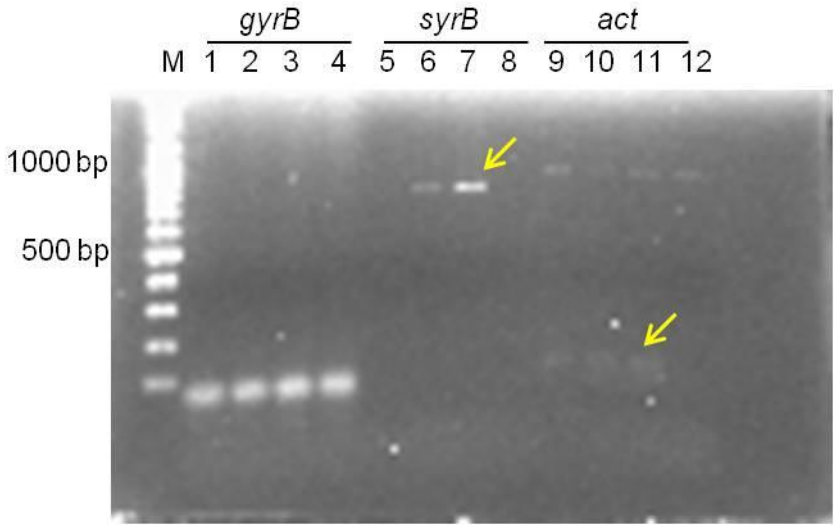

Figure 2-11. PCR detection of syrB expression in Duke leaf discs inoculated with Pss9, Pss9R, or mock-inoculated.

Total RNA extracted from inoculated discs served as a template for cDNA synthesis using random oligonucleotides as primers and reverse transcriptase. Resulting cDNA was then amplified by PCR using primer pairs for bacterial DNA gyrase (*gyrB*), syringomycin synthetase (*syrB*) or plant actin (*act*) and PCR products for each primer pair were loaded onto the gel as indicated. M is the 10 Kb O'geneRuler DNA ladder (Fermentas). Yellow arrows indicate bands of expected size for the amplicon indicated. Lanes 1, 5, and 9 are mock-inoculated controls. Lanes 2, 6 and 10 are Pss9R-inoculated discs. Lanes 3, 7 and 11 are Pss9-inoculated discs. Lanes 4, 8 and 12 are PCR master mix blanks.

Pss and *P. viridiflava* were detected most frequently on blighted blueberry stems. A more intensive survey of the microbial community associated with blueberry tissues would be required to determine whether Pss is the predominant strain. The role of *P. viridiflava* in bacterial blight of blueberry was not determined. Given its high frequency of isolation in this study, it may be a common invader of lesions induced by Pss or it may have a more active role in the disease. *P. viridiflava* has not been reported as a pathogen of blueberries but it is pathogenic on annual crops such as beans, onions, and crucifers (Bradbury, 1987) and has a potentially wider host range as a leaf-spot, stem-rot, and leaf-necrosis agent on many other herbaceous annuals (Goumans and Chatzaki, 1998). *P. viridiflava* as the causal organism of apple blossom blight has been reported recently in Africa (Alimi *et al*., 2011). *P. viridiflava* strains have been reported to nucleate ice (Jakob *et al*., 2002; Anderson and Ashworth, 1986) and some were found to be capable of producing levan, which enhanced virulence on kiwi (Gonzalez *et al*., 2003). It is notable that *P. viridiflava* has never been associated with bacterial blight symptoms on any other woody hosts in the Fraser Valley.

Other Biolog-identified pathovars of *P. syringae* isolated from blueberry are known to be pathogens of herbaceous ornamentals such as marigold, sunflower and snapdragon (pvs. *tagetis*, *helianthus* and *antirrhini,* respectively) and field crops such as celery and sugar beets (pvs. *apii* and *aptata,* respectively). These pathovars may be present in blueberry fields as a result of weedy hosts such as dandelion and thistle (Compositae), toadflax (Scrophulariaceae), Queen Anne's lace (Umbelliferae), pigweed and lamb's quarters (Chenopodiaceae) which have been reported as reservoirs for both *P. syringae* (Baca and Moore, 1987; Kinkel *et al*., 1996; Malvick and Moore, 1988;) and *P. viridiflava* (Mariano and McCarter, 1993). Alternatively, these phytopathogenic *Pseudomonas* strains may be part of the microbial community on blueberry tissues and may contribute to disease development under favourable circumstances. Isolates of P.s. pv.*apii*, *tagetis* and *helianthi* were not pathogenic on lilac; however, pvs. *antirrhini* and *aptata* were not tested.

Only Pss and *P. viridiflava* isolates were pathogenic on lilac plantlets. Although lilac is a woody shrub, tissue cultured plantlets were neither highly lignified nor suberized and leaves and stems remained tender. *P. viridiflava* has been reported to produce pectate lyases responsible for inducing soft rots on its typical herbaceous hosts (Liao *et al.*,

1998) and lilac infected by *P. viridiflava* displayed symptoms consistent with soft rot syndrome. In contrast, Pss produced spreading lesions. Two isolates of Pss did not produce any symptoms on lilac; however, the original Pss type strain from lilac (NCPPB 281 = ATCC 19310) also failed to produce symptoms. This isolate was reported to be weakly virulent on lilac in 1972 (ATCC catalogue comment), and was avirulent when inoculated on wounded lilac leaf lamina at 10^7 CFU/mL and virulent when inoculated into petioles and midribs at 10^8 CFU/mL (Young, 1991). This strain may have become attenuated through multiple generations on laboratory media (levan production also was lacking) or it may be particularly weak by nature. It is possible that different Pss isolates from blueberry also exhibit a natural range of virulence.

The effect of population size on disease development was examined with lilac plantlets inoculated with a virulent Pss isolate under laboratory conditions. Progression of disease was more variable at low doses compared with higher doses of Pss and disease curves plateaued for low doses on wounded lilacs. It was expected that small doses of Pss would multiply on plantlets as efficiently as large doses, and that they would face reduced competition for nutrients and space initially. Because bacteria were not enumerated at the end of the serial dilution inoculation trials, it was not known whether plantlets with low doses and less severe disease actually had fewer bacteria than plantlets with high doses. Differences in the host's ability to restrict the proliferation and virulence of small bacterial aggregates vs. large ones may have been a factor.

Dulla and Lindow (2008) demonstrated that quorum sensing (QS) in Pss can be induced in 4 days in aggregates as small as 22 cells on wet leaf surfaces and that induction was even faster on dry leaf surfaces. The Magenta jars used in this study provided high-humidity environments and water droplets from either wounding or dipping inoculations remained visible on plantlet surfaces for 24-72 h. Pss has been reported to use *N*-acyl-homoserine lactones (AHLs) as autoinducers (Dumenyo *et al*., 1998; Quinones *et al*., 2005) which may diffuse in water. Alternatively, excess water may have delayed *P. syringae* adhesion to surfaces (Morisaki, 1995).

For the wounded lilacs, the two least concentrated inocula that induced symptoms were estimated to have 100 and 1000 CFU, respectively; these numbers should have been sufficient to induce QS on ostensibly wet plantlets. Involvement of QS in Pss virulence

has been shown through induced alginate production and reduced sensitivity to oxidative stress (Quinones *et al*., 2005). In addition, plant signal molecules such as phenolic glycosides and sugars (sucrose, fructose and mannose) have been shown to induce virulence factors, specifically syringomycin, in Pss isolates (Mo and Gross, 1991a; Mo *et al*., 1995). Given that the quorum was met and plant compounds were presumably released through wounding, it is not known why symptoms did not spread to a greater extent within 14 days when inoculated with low doses. Prolonged incubation may have resulted in eventual plantlet death but this was not determined in the current study.

Pathogenicity assays on blueberry leaf discs revealed that host tissue response to wounding in the absence of the pathogen changed over the growing season, as indicated by increased marginal blackening which was likely due to defensive oxidation and accumulation of phenolics at the wound site. Damage-associated molecular patterns (DAMPs) are known to trigger plant defenses upon recognition of self-injury (reviewed in Boller and Felix, 2009). Control discs cut from Duke leaves picked in late May responded to wounding by initiating formation of healthy callus along the disc perimeter. In mid-June, Duke control discs began to exhibit necrosis at the margin, and by mid-July necrosis at the margin became more pronounced. This boost in host response to wounding may be responsible for the relative reduction in Pss-induced necrosis, failure of disease to progress from 3 to 5 dpi and the drastic decline in re-isolated bacteria in the July trial. However, necrosis due to inoculation remained significantly greater than that seen in controls, which suggested that plant responses elicited by pathogen-associated molecular patterns (PAMPs) in combination with DAMPs are stronger than those elicited by DAMPs alone. Bacterial PAMPs are known to initiate a cascade of plant defenses (reviewed in Bent and Mackey, 2007).

Age-related resistance (ARR) has been demonstrated in plant-bacteria interactions between rice and *Xanthomonas campestris* pv. *oryzae* (Koch and Mew, 1991; Mazzola *et al*., 1994; Century *et al*., 1999) and *Arabidopsis thaliana* and *P. syringae* pvs. *tomato* and *maculicola* (Kus *et al*., 2002). ARR has been shown to limit bacterial populations on resistant and susceptible rice leaves (Mazzola *et al*., 1994) which may be related to plant developmental stage (ontogeny) rather than age of the leaf itself (Century *et al*., 1999). Greenhouse-grown Arabidopsis ecotypes resistant to bacterial speck can restrict bacterial proliferation *in planta* and also display ARR-like responses when subjected to

environmental conditions that force ontogenic changes such as precocious maturation (Kus *et al*., 2002; Rusterucci *et al*., 2005). In relation to pathogen resistance, little work on ARR has been conducted on woody plants and never on blueberry to our knowledge. The phenological stage of field-grown blueberries at the times of leaf selection may have contributed to ARR to Pss.

The same effects were seen in Elliott leaf discs as described for Duke, but they were even more pronounced in that both greater necrosis and restriction of bacterial growth occurred on Elliott. Elliott is a late-blooming cultivar that lags behind Duke by up to 4 weeks depending on growing-degree days, a measure of heat accumulation, for a given year. Elliott buds remained tight while Duke was leafing out in the year that the disc assay was conducted. Late-developing cultivars would be exposed to greater amounts of herbivory when they finally leaf out as insect pests will have established in the field. Late bloomers may therefore need to upregulate active defenses quickly and to a larger degree to protect foliage. This is especially true in blueberry fields since it is a common practice to extend the productive season by planting bushes in adjacent blocks of early-, midseason- and late-blooming varieties. Aside from increased herbivory, late-blooming plants may escape pests that proliferate early in the season such as fungi that release ascospores in spring and possibly Pss itself. Because Elliott buds are not released from dormancy during periods when damaging frosts are most likely to occur, these tissues remain protected.

Differences between Elliott and Duke in the magnitude of response to bacterial blight may reflect true resistance in Elliott rather than just temporal effects. Elliott is resistant to the fungal diseases mummy berry (Eck, 1998; Gough, 1994) and anthracnose (Miles *et al*., 2011) and has been noted to be resistant to bacterial blight in field; however, this last observation is not based on controlled inoculation studies (Pscheidt and Ocamb, 2008; Caruso and Ramsdell, 1994). Elliott shares more germplasm with other blight-resistant cultivars than does Duke (Gough, 1994). Similarly, susceptible and resistant cultivars of pear (Deckers and Schoof, 2001) and cherry trees were shown to differ in the numbers of epiphytic Pss harboured (Burkowicz, 1981; Crosse, 1963).

The mutant strain Pss9R was found to be deficient both in the ability to induce necrosis and the ability to colonise leaf tissue when compared to wild type Pss9. Reasons for the

attenuation of virulence or inability to cope with host defenses in either Duke or Elliott leaf disks were not investigated.

Syringomycin was found to be active *in planta* at the nanomolar scale (Hutchison and Gross, 1997). The mechanism of syringomycin toxicity is pore formation in plasma membranes of both fungal and plant cell, causing cytolysis (Hutchison and Gross, 1997; Hutchison *et al*., 1995; Dalla Serra *et al*., 1999). Syringomycin also has been shown to complex with fungal cell wall constituents such as β-1,3-glucan, chitin, mannan and sterols (DeLucca *et al*., 1999). In this study, crude extracts from Pss cultures grown *in vitro* had fungistatic properties. When autoclaved, the extracts retained some activity against fungal growth which suggested that either non-heat labile compounds in the extraction or thermal breakdown products of syringomycin also possess antifungal activity. Alternatively, autoclaved extracts may have had undetectable amounts of intact syringomycin that were sufficient to inhibit fungal growth.

How syringomycin penetrates the plant cell wall to reach the plasma membrane has not been described. SyrD, an ATP-binding cassette (ABC) transporter protein exports the toxin (Quigley *et al*., 1993) and may also help prevent auto-toxicity by efficient efflux pumping (Grgurina *et al*., 1996). Penetration to the host plasma membrane may be aided by enzymatic breakdown of plant cell wall components and Pss B728a was found to have genes encoding cellulases, pectate lyase, and xylanase (Feil *et al*., 2005; Lindeberg *et al*., 2008). Detection of *syrB* expression in inoculated lilacs and blueberry leaf tissue suggested that Pss9 was competent in producing virulence factors during interaction with the host.

Production of syringomycin and related toxins has been reported to occur in putatively saprophytic isolates of *P. syringae* (Adetuyi *et al*., 1995). The authors concluded that toxin production does not correlate with pathogenicity; however, no evidence of the hypersensitive response (HR) was provided. HR would be expected with inoculations of high numbers of bacteria into non-host plants. Some Pss strains have been shown to retain pathogenicity on hosts when toxin synthesis is knocked out (Scholz-Schroeder *et al*., 2001; Xu and Gross, 1988) while other Pss isolates naturally do not produce toxins (Bultreys and Kaluzna, 2010). The latter is also true for other pathovars, such as

phaseolicola which normally produces phaseolotoxin (Rico *et al*., 2006). Syringomycin is therefore significant for Pss virulence but not required for pathogenesis.

Syringomycin may serve multiple functions. The contribution of syringomycin to epiphytic fitness has been demonstrated previously (Beattie and Lindow, 1999; Sundin, 2006) and *Pseudomonas* has been reported to have a prolonged epiphytic stage in its association with host plants (Beattie, 2006). Syringomycin has been demonstrated to act as a biosurfactant for retention of water films on leaves (Hutchison *et al*., 1997; Dalla Serra *et al*., 1999), normally a very hydrophobic surface and induction of syringomycin synthesis occurs on leaf surfaces (Marco *et al*., 2005, Volksch and Weingart, 1998).

The presence of Pss on blueberry plants in the Fraser Valley and its demonstrated pathogenicity on lilac and blueberry tissue in laboratory testing confirm its potential role in bacterial blight. The pathogenicity of a Pss isolate was tested under field conditions in studies reported in Chapter 3.

3. Epiphytic survival and molecular detection of *Pseudomonas syringae* on blueberry plants

3.1. Introduction

Bacterial blight caused by *Pseudomonas syringae* pathovar *syringae* van Hall (Pss) is a disease that sporadically affects commercial blueberry (*Vaccinium corymbosum* L.) fields in the Fraser Valley of British Columbia, one of the largest areas of production worldwide. Blight symptoms include brown-black stem cankers, bud blight and tip dieback and primarily affect tender one-year old tissues. Reduction in yield due to damaged floral buds and stem girdling occurs in years with severe disease outbreaks and productivity of young fruit orchards may be delayed by damage to scaffold branches and generative buds.

Pss is a ubiquitous, generalist pathogen with a very broad host range encompassing both herbaceous annuals and woody perennials. Many orchards of fruit- and nut-bearing trees throughout Europe, Africa, Australia, Asia and South and North America are subject to bacterial blight. The diseases are typified by necrotic lesions on susceptible tissues. Examples include stem canker and bud blight in cherry (Foulkes and Lloyd, 1980; Hattingh *et al*., 1989; Li and Sholberg, 1992; Latorre *et al*., 1985), and blossom blast, stem canker and dieback in peach (Endert and Ritchie, 1984; Little *et al*., 1998), plum (Hinrichs-Berger, 2004) and apricot (Hattingh *et al*., 1989). Pome and stone fruit trees are among the best-studied woody hosts of Pss given their economic importance but many ornamental trees and shrubs common in nurseries and landscaping such as lilac, magnolia, poplar and willow are also hosts in the Pacific Northwest (Pscheidt and Ocamb, 2008).

Pss has been described as a weak or opportunistic pathogen because of its inability to enter plant tissues except through natural openings or wounds (Agrios, 1997). Phytopathogenic bacteria that occupy the phylloplane commonly use stomata, lenticels

and wounds such as broken trichomes and excision scars from leaf and bud scale dehiscence to access the apoplast (reviewed in Huang, 1986). Herbivory, weather-related events such as hail damage, abrasion by wind-driven grit and frost cracking and horticultural practices such as pruning and harvesting also produce wounds which allow entry of bacteria (Huang, 1986).

In addition to its weak pathogenicity, Pss has a prominent epiphytic phase in its life cycle. This has been studied extensively on leaves of herbaceous annuals in temperate regions during the growing season (Beattie and Lindow, 1994a; Hirano *et al*., 1982; Hirano and Upper, 2000; Kinkel *et al*., 1996; Monier and Lindow, 2004; Schreiber *et al*., 2005; Voloudakis *et al*., 1991; Wilson and Lindow, 1993; Wilson *et al*., 1999) and less commonly on leaves or blossoms of woody perennials (Crosse, 1963; Latorre *et al*., 1985; Lindow and Andersen, 1996; Stockwell *et al*., 1999). Resident epiphytic populations of Pss are continually in contact with the host but merely persist on plant surfaces without causing disease (Hirano and Upper, 1990; Beattie, 2006). Bacteria must gain entry before disease symptoms are expressed.

Leaves colonized by Pss may contribute to the inoculum for stem infections as they dehisce at the end of the growing season (Crosse, 1966; Kennelly *et al*., 2007) and resident stem populations fluctuate seasonally (Endert and Ritchie, 1984). Pss can overwinter on its hosts and perennial lesions may serve as inoculum sources for new infections both within and between hosts (Kennelly *et al*., 2007). Dissemination of phytopathogenic bacteria through orchards has been demonstrated previously (Lindow and Andersen, 1996; Quesada *et al*., 2010). Immigration via atmospheric aerosols created by wind-driven rain increases epiphytic populations of both phytopathogenic and non-pathogenic bacteria on hosts (Bock *et al*., 2005; Lindemann and Upper, 1985) as well as non-host reservoirs such as weeds and cover crops (Baca and Moore, 1987; Kinkel *et al*., 1996; Malvick and Moore, 1988). This may be of importance for disease control as several investigators have failed to detect Pss on hosts but have detected it on nearby weeds (Malvick and Moore, 1988).

To date, no investigations have been undertaken to clarify factors associated with bacterial blight outbreaks in blueberry fields. An understanding of Pss population dynamics would aid in the effective management of bacterial blight. A previous survey in

the Fraser Valley showed that resistance to fixed copper formulations, currently the only chemical bactericides available for blueberry growers, was widespread (MacDonald *et al.*, 2002). Refined timing for application of chemical controls and rotation with alternative biological controls can help reduce oversprays, preventing the further spread of resistance. Furthermore, a better understanding of the epidemiology of bacterial blight on blueberries could be used to develop recommendations to growers on timing of bactericide applications.

The purposes of this research were to: (i) monitor inoculated populations of Pss on blueberry plants in the field from fall to spring; (ii) relate populations to environmental variables and disease incidence; (iii) develop a rapid screening method to distinguish Pss from other *Pseudomonas* species and pathovars isolated from field samples.

3.2. Materials and methods

3.2.1. Epiphytic survival of Pseudomonas

3.2.1.1. Production of inocula

For the field trials described below, inoculum was produced as follows. Frozen, stored cells of isolate Pss9 and its derived rifampicin-resistant strain Pss9R (described in Chapter 2) were transferred from cryovials (Nalgene, Rochester, NY) with a sterile toothpick to 10 mL LB in 30 mL screw-cap tubes. These were grown for 24 h at RT and 100 rpm on an orbital shaker (Barnstead Thermolyne, Dubuque, IA). Each turbid culture was then used to inoculate a set of five 250 mL flasks containing 100 mL LB. These were incubated for 48 h under the same conditions. The optical density of a 1 mL aliquot from the 5 combined flasks of each culture was measured on a spectrophotometer (Novaspec II, Pharmacia LKB) at λ = 590 nm and a second aliquot was used for serial dilution plating to enumerate CFU and confirm culture purity. Cells were pelleted by centrifugation at 8000 rpm, 12°C, for 10 min (Sorvall RC6, SLA-1500 SuperLite rotor, Mandel Scientific), washed with PBS and re-pelleted. The final pellets were resuspended in 30 mL PBS and transferred to Falcon tubes for transport to the field as concentrated inocula held on ice in a cooler.

3.2.1.2. Field design and bacterial application

Ninety 2-year old potted blueberry plants cv. Duke and Bluecrop, two of the most common cultivars in the Fraser Valley, were grown in 1 L pots by a commercial propagator in fall, 2008. On December 2, 2008, 15 plants of each cultivar were randomly assigned to each of the following treatments: buffer-sprayed control (PBS), Pss9 spray-inoculated (10^7 CFU/mL), and Pss9R spray-inoculated (10^7 CFU/mL). Plants were sprayed individually with a hand-sprayer until run-off (approximately 30 mL) and then held in spatially separated groups during staging. Plants remained grouped overnight and then each was randomly assigned a 1 m^2 area on the ground cover the following day. Inoculum was checked for viability by plating after return to the laboratory.

The field trial was repeated in fall, 2009 except that only the Duke cultivar was used with increased replications, inocula were combined into one treatment, and a wounding variable was included. A single cultivar was used because no differences between cultivars were found in the first year. One hundred and sixty 2-year old Duke plants were randomly assigned to one of four treatments: buffer-sprayed control, buffer-sprayed control with wounding, Pss9 + Pss9R-sprayed, Pss9 + Pss9R-sprayed with wounding. Strains Pss9 + Pss9R were applied in a 1:1 (v/v) ratio. Wounding consisted of stabbing a #5 insect mounting pin into young stems and pulling the pin in a downward motion to scratch through the epidermis. Each stem was stabbed 10 times such that scratches formed a non-continuous line down the stem starting at the tip. All the tender stems of the current year's growth were wounded, with a minimum of 3 stems wounded per plant. Plants were sprayed until run-off (approximately 30 mL) with PBS or a 10^6 CFU/mL cell suspension in PBS. Plants were moved onto the ground cover the day following treatment but remained grouped in 4 spatially separated blocks, approximately 5 m apart. Plants were inoculated on October 23, 2009 and re-inoculated one week later on November 2 to ensure that Pss populations could establish themselves on the plants in the field. Leaves had not dehisced by the date of the second inoculation.

An Environment Canada weather station was located on site in Abbotsford, BC approximately 50 m away from the field plots. Weather data for the duration of both field trials were accessible from a public website. Daily high and low temperatures, total

precipitation, and wind speed and direction were downloaded as well as 30-year weather averages.

3.2.1.3. Recovery of bacteria

In the first year of the field trial, each plant was sampled every 2 weeks from December, 2008 to May, 2009 by removing 1-centimeter sections from the tip of 3 twigs. Cuttings were bulked as a single sample per plant. Consecutive samples were taken from the same twigs on each sampling date. Samples were collected and returned to the laboratory in a cooler. The sections of stem tissues and any associated buds were shaken in 10 mL sterile PBS for 1 min to dislodge epiphytic bacteria. The resulting wash was serially diluted and plated in duplicate on KBC. A subset of samples was duplicate-plated on NA. Twig sections were then surface sterilized by immersion in a 2% NaOCl solution with 0.01% Tween 20 for 1 min followed by a triple rinse in SDW. Samples were then allowed to dry in a laminar flow hood and macerated as previously described. Macerate was serially diluted with SDW and plated in duplicate on KBC, with a subset of samples on NA to recover internal bacteria. It should be noted that surface sterilization may not be sufficient to remove epiphytically adhered bacteria protected by biofilms and, therefore, populations described as “internal” may be a combination of epidermal and subepidermal cells. Resulting colonies were counted after 3-5 days incubation at RT and CFU per gram tissue fresh weight was calculated for each sample.

In the second year of the field trial, 5 plants of each treatment were sampled every 4 weeks from November, 2009 to May, 2010. A 1-cm section of stem tissue from the tip down was sampled from each young stem and all sections from a single plant were bulked as one sample. Sampled tissue areas did not extend below the zone of wounding for plants with that treatment, as wounding scars were easily visible throughout the sampling period. Only internal bacteria were recovered after surface-sterilization by plating tissue macerates in the second trial.

3.2.2. Molecular detection of Pss

A Southern blotting method was developed to accurately and rapidly distinguish Pss colonies from non-Pss colonies that grew out from dilute tissue macerates plated on KBC in the second year of the field trial. Since KBC is only semi-selective for Pss and

the rifampicin-resistant strain did not persist in high numbers under field conditions in the first year, DNA-DNA hybridization was used to probe and identify multiple colonies on individual plates simultaneously. Genes involved in toxin production have been used for *P. syringae* pathovar-specific probe development previously (Cuppels and Elmhirst, 1999; Scheck *et al*., 1998).

After incubation and enumeration, colonies were lifted from selected agar plates within a range of 30-100 colonies using Hybond® XL nylon membranes (GE Healthcare Life Sciences) cut to fit 100 x 15 mm Petri dishes. Adhered colonies were lysed and the released DNA was bound to membranes using the following protocol: lysis for 3 min (10% SDS), denaturation for 5 min (0.5 N NaOH, 1.5 M NaCl), neutralization for 5 min (0.5M Tris-HCl, 1.5M NaCl, pH 7.4) and rinse for 5 min (2x SSC). Membranes were treated by laying them colony-side up on a large piece (25 x 50 cm) of Whatman No. 1 filter paper in a series of four trays filled with solution sufficient to soak the filter paper (approximately 50 mL). After the required treatment time had elapsed, membranes were transferred from one tray to the next in the series. Up to 12 membranes could be processed simultaneously in this manner. DNA was cross-linked to membranes with a UV Stratalinker 2400 (Stratagene, La Jolla, CA) set to auto-crosslink and colony debris was removed by submerging membranes in washing solution (5x SSC, 0.5% SDS, 1 mM EDTA pH = 8) and gently agitating on a rocking platform shaker (LabLine MAXI rotator, Barnstead Thermolyne, Melrose Park, IL) for 30 min at 50°C. Membranes were then rinsed with 2x SSC and stored at -20°C wrapped in paper towels soaked in 2x SSC and sealed in plastic bags.

The hybridization probe was based on the 756 kb *syrB* gene fragment amplified by the B1/B2 primer pair. A new primer pair was designed using Primer3 v. 0.4.0 (Rozen and Skaletsky, 2000) which annealed within the *syrB* sequence to create a shorter amplicon (222 bp) for increased specificity during DNA-DNA hybridization. Template DNA was generated by 30 cycles of PCR using the same steps as described for *syrB* amplification except that the annealing temperature was changed to 52°C and 33 cycles were run. PCR products were loaded onto agarose gels as previously described to check for single, specific amplicons of the expected size. The template DNA was purified using a QIAquick PCR purification kit or gel excision kit (QIAgen Inc) and its concentration was quantified using a NanoDrop 2000c spectrophotometer (Thermo Scientific). Radioactive

probe was synthesized with random oligodeoxyribonucleotides (Invitrogen) as primers and the Prime-a-Gene® labelling kit (Promega, Madison WI) to incorporate [α-^{32}P] dCTP as per the manufacturer's instructions and purified using Illustra MicroSpin® S-200HR columns or Illustra ProbeQuant G-50 Micro columns (GE Healthcare) as per the manufacturer's instructions.

Membranes were sealed into hybridization tubes (35 mm ID x 300 mm) with 30 mL EKONO™ hybridization buffer (Research Products International Corp., Mt. Prospect, IL) for 1 h in a hybridization oven set to 60°C and 6 rpm prior to addition of the radioactive probe. Hybridization was performed overnight in the oven at 65°C and 6 rpm followed by 4 washes with 2x SSC+0.1% (w/v) SDS at 60°C, the first wash for 5 min and subsequent washes for 10 min. Washed membranes were rinsed in 2x SSC, enclosed in plastic wrap, then placed in autoradiography cassettes (Kodak) containing a blanked storage phosphor screen (GE Healthcare) for exposures of 4-16 h at RT. Exposed screens were then visualized using a Typhoon 9410 variable mode imager (Molecular Dynamics, Sunnyvale, CA) using the 390 BP filter.

Prior to use, probe specificity was checked by plating assorted bacteria in non-random patterns on single agar plates. Bacteria included non-pseudomonads such as *Erwinia amylovora*, *Escherichia coli*, *Stenotrophomonas maltophilia*, *Bacillus subtilis*, and *Pantoea agglomerans* and non-*syringae Pseudomonas* strains such as pv. *viridiflava* and *aeruginosa*. All *P. syringae* pathovars previously identified with Biolog were also screened, including putative Pss isolates.

3.2.3. Statistical analyses

For all experiments, data were analysed with Statistical Analysis System v.9.2 software (SAS Institute, Cary IN). Normality of all data was examined with *proc univariate* and the appropriate parametric test or its non-parametric equivalent was selected for further analysis. The type 1 error rate was set at $\alpha = 0.05$ for all tests.

Results from the two inoculated field trials were analysed separately. Numbers of recovered pseudomonads were log-transformed (y +1) prior to analysis in all cases. Data for year 1 were analysed with *proc ttest* for comparisons of recovered bacteria from control plants and Pss9 inoculated plants. Plants inoculated with Pss9R were not

included in the analysis due to extremely low recovery. For year 2, analysis of variance was performed for the 2x2 factorial experiment with *proc GLM* by including both variables and interactions in the model statement. Pairwise comparisons of treatments were performed with Tukey's HSD test.

Proc corr was used to perform Pearson product-moment correlational analysis of recovered CFU/g and weather variables for each field year. Mean maximum and minimum temperatures and cumulative precipitation were calculated for the 14-, 7-, 4-, and 2-day intervals preceding each date of sampling and these means were correlated with recovered CFU/g for each treatment. Possible significant differences between *r* values for these independent correlations of weather and bacterial populations by treatment were determined with the Fisher *r*-to-*z* transformation using the *fisher* option for *proc corr*. *Proc corr* also was used to analyse the possible correlation between the number of buds associated with twigs and recovered CFU/g.

The proportion of pseudomonads to all bacteria recovered from blueberry stems was calculated by comparing mean CFU/g recovered on selective medium (KBC) vs. non-selective medium (NA). Five tissue samples for each sampling date and each treatment were plated on both media for both years of the study. The average CFU/g recovered on NA was set to equal 1 and the average CFU/g recovered on KBC was a proportional value relative to that.

For year 2 data, the percentage of hybridization-positive CFUs was calculated by averaging positive colonies and total colonies for each sampling date and treatment. Number of samples for each calculation was variable due to use of plates only within a specific counting range. An analysis of variance between all treatments was performed with *proc GLM* and pairwise comparisons were performed with Tukey's HSD.

3.3. Results

3.3.1. Epiphytic survival

Individual blueberry plants had highly variable numbers of pseudomonads present, ranging from zero (undetectable) to 10^7 CFU/g on a given sampling date. Despite this,

the population mean fluctuated with seasonal progression whether bacteria were epiphytic or internalized (Fig. 3-1). Fewest bacteria were recovered in late December, 2008, which coincided with the coldest period of the season (appended Fig. A3-1). Epiphytic *Pseudomonas* populations were as low as $10^{0.52}$ CFU/g on controls and $10^{0.50}$ CFU/g on Pss9-treated plants; however, internal populations were somewhat larger. For example, controls had $10^{0.92}$ CFU/g and Pss9-treated plants had $10^{1.2}$ CFU/g at this same sampling date. Epiphytic populations showed an approximately 5-fold increase as early as mid-January, 2009, on both groups of plants while internal populations increased 5.8-fold (controls) or 3.4-fold (Pss9-inoculated) during this period.

Maximum recovery of epiphytic *Pseudomonas* occurred in mid-March for controls ($10^{4.6}$ CFU/g) and late April ($10^{4.7}$ CFU/g) for Pss-9 inoculated plants. Maximum internal *Pseudomonas* populations were found in early May for both control ($10^{6.7}$ CFU/g) and Pss9 plants ($10^{6.5}$ CFU/g). Numbers of *Pseudomonas* recovered from control and inoculated plants were not significantly different during the first year of the field trial except in two instances. These exceptions were mid-January internal populations (p<0.001) and late February epiphytic populations (p=0.004).

Mean recovery of Pss9R was very low (< 10^1 CFU/g) at all sampling times and when re-isolated, levels were found more frequently as internal populations than epiphytes, indicating that the mutant strain was capable of gaining entry to the blueberry tissues (Fig. 3-2). Only 13 of 30 replicates yielded Pss9R, and 4 of these plants had detectable levels of Pss9R at 4 or 5 sampling times beginning from mid-January, 2009. Also, on two dates during the latter half of the field sampling period (late February and early May), internal and epiphytic populations of Pss9R were detected concurrently on 3 plants. These few plants appear to have become chronically infected by Pss9R. There was nothing distinctive about these plants such as order in which they received inoculation, allocated position on the ground cover, exposure, or cultivar. Taken in isolation, the maximum epiphytic recovery from these chronically infected plants reached 10^3-10^4 CFU/g in early May and maximum internal populations of 10^5 CFU/g on the same date. Pss9R-inoculated plants were comparable to control and Pss9-inoculated plants in terms of total bacteria recovered as epiphytes or internal populations (Fig. 3-3).

Total culturable bacteria (Fig. 3-3) on and in blueberry stems were enumerated by

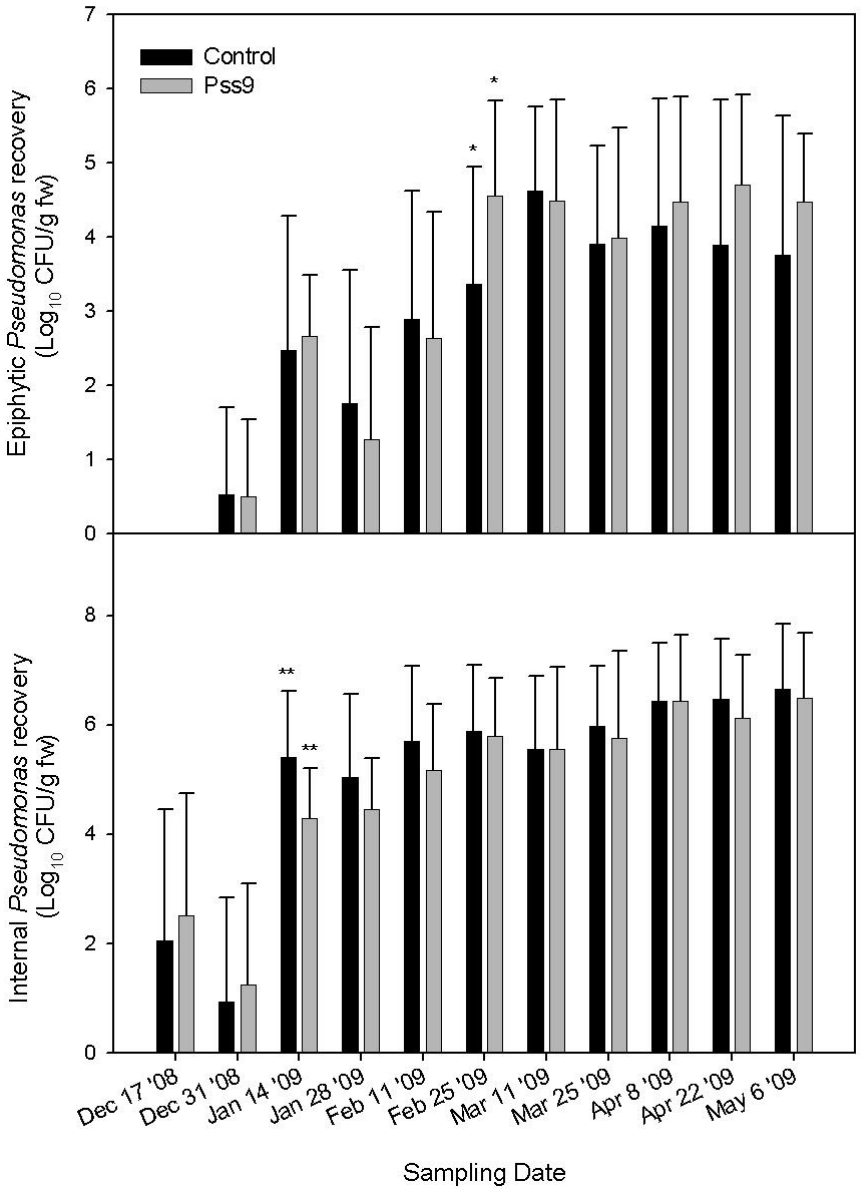

Figure 3-1. Recovery of epiphytic and internal pseudomonad populations from control and Pss9-inoculated blueberry stems during year 1 of the field trial.

Bars indicated with asterisks are significantly different at that sampling date (α = 0.05). Log values for mean CFU/g fresh weight and standard deviation were calculated for 30 samples. Error bars represent standard deviation. Data were missing for December 17 epiphytes.

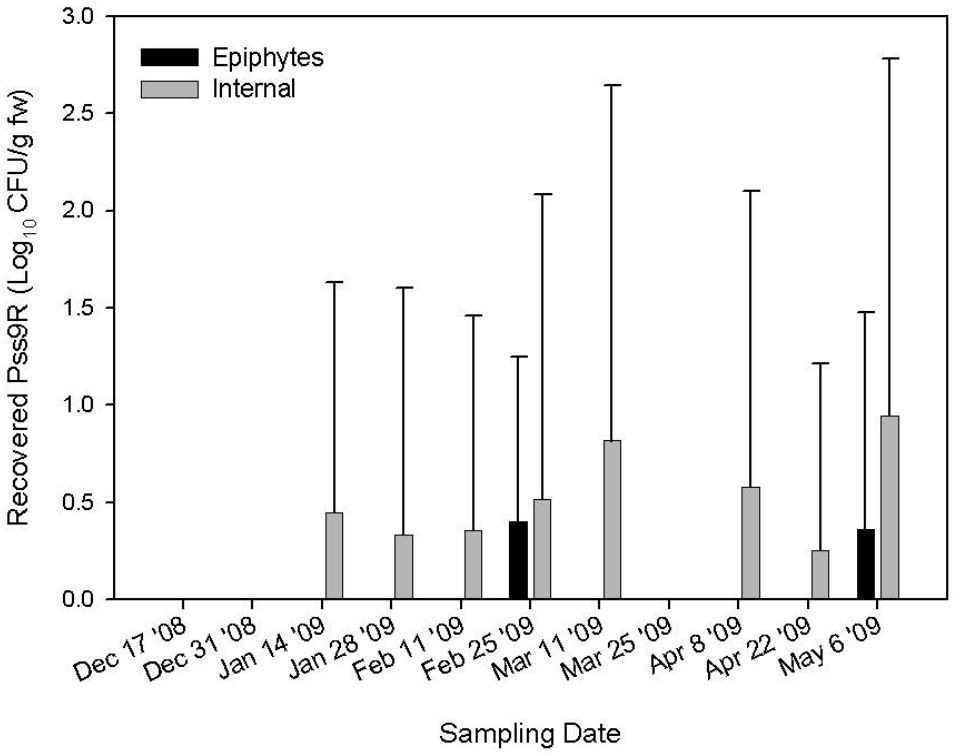

Figure 3-2. Recovery of Pss9R from inoculated blueberry stems as an epiphytic and internal population during year 1 of the field trial.

Log values for mean CFU/g fresh weight and standard deviation were calculated for 30 samples. Error bars represent standard deviation.

plating on a non-selective medium. Maximum epiphytic levels on controls were $10^{4.8}$ CFU/g in early April, and were $10^{5.8}$ and $10^{5.3}$ CFU/g for Pss9 and Pss9R plants in late February. Total internal bacterial levels on controls, Pss9, and Pss9R-treated plants reached a maximum $10^{6.8}$ (early April), $10^{7.1}$(early May), and $10^{6.5}$ (late February) CFU/g respectively. Pseudomonads comprised an increasing proportion of the total epiphytic population as winter progressed into springtime, but were less prone to seasonal fluctuation compared to internalized populations (Fig. 3-4). On controls, pseudomonads reached a maximum proportion of 66% of total epiphytes in early May and a maximum of 49% of internal bacteria in early February. The maximum epiphytic proportion of pseudomonads on Pss9-treated plants (51 %) also occurred in early May and the maximum internal proportion in early April was 37%. Differences in magnitude and chronology of *Pseudomonas* levels in control and treated plants may be due to the variability seen in the numbers of *Pseudomonas* found on individual blueberry plants.

There were no differences between the two cultivars in the number of recovered bacteria either epiphytically or internally for any treatment (data not shown). The number of buds associated with twigs macerated for bacterial detection showed no correlation to numbers of *Pseudomonas* recovered (data not shown).

A moderate correlation was found between temperature and epiphytic or internal *Pseudomonas* populations over the duration of the field trial. The maximum daily temperature averaged over *n* days preceding the sampling date for up to 14 days was positively correlated with numbers of recovered *Pseudomonas* (Fig. 3-5). Correlations were stronger for internal populations than for epiphytes in both control (r = 0.649, 0.439) and Pss9-treated plants (r = 0.67, 0.624). Positive correlations were also found for 7-, 4-, and 2-day temperature averages but became weaker as the average was taken over fewer days (data not shown).

Moderate correlations also were found between *Pseudomonas* populations and cumulative precipitation. Total precipitation was calculated for the same pre-sampling time intervals and was found to have a negative relationship with both epiphytic and internal population sizes which was strongest with 4-day averages (Fig. 3-6).

Independent correlations of temperature variables and bacterial populations were

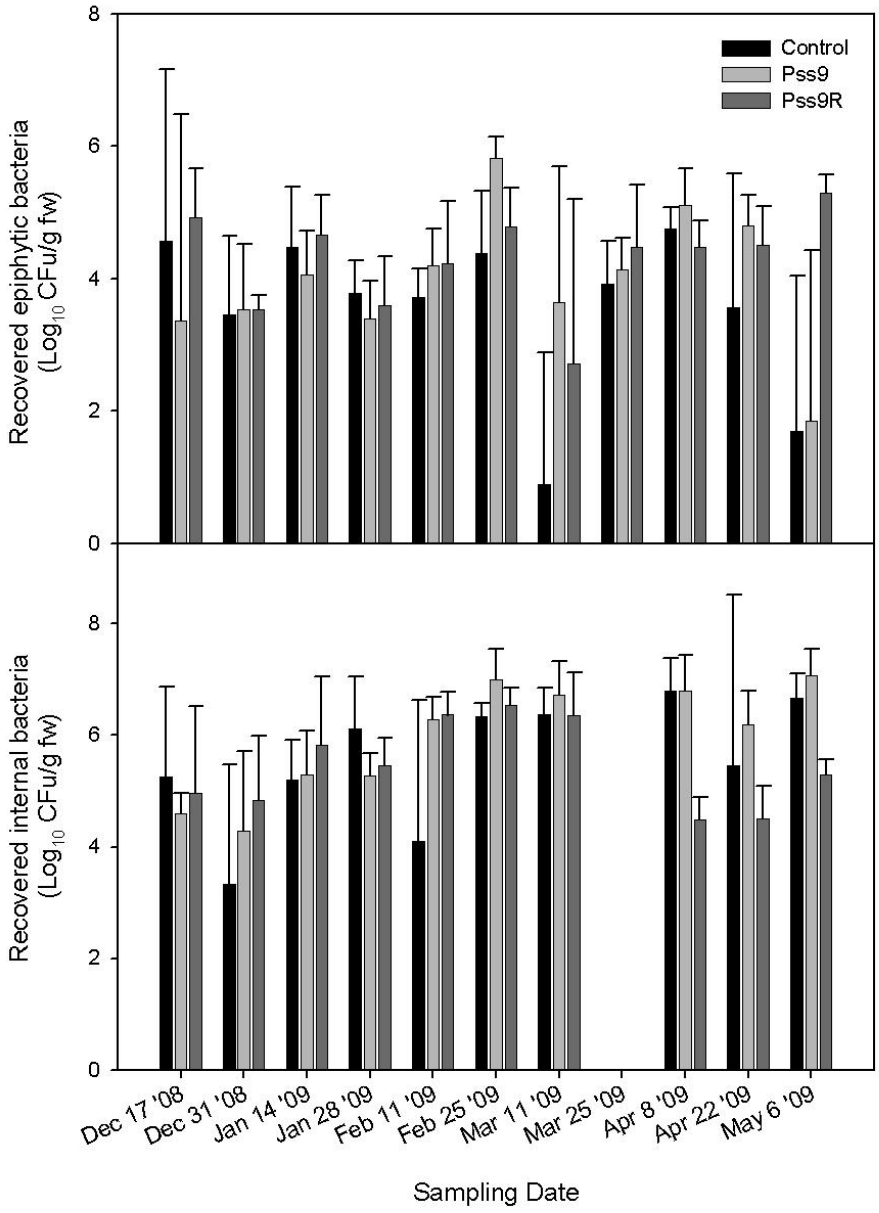

Figure 3-3. Total culturable epiphytic and internal bacteria recovered from control, Pss9-inoculated and Pss9R-inoculated blueberry stems during year 1 of the field trial.

Epihytic populations are represented in the top panel and internal populations are represented in the bottom panel. Means and error for each treatment were calculated from 30 replicates for each sampling date. Error bars represent standard deviation. Data for internal populations are missing for March 25, 2009.

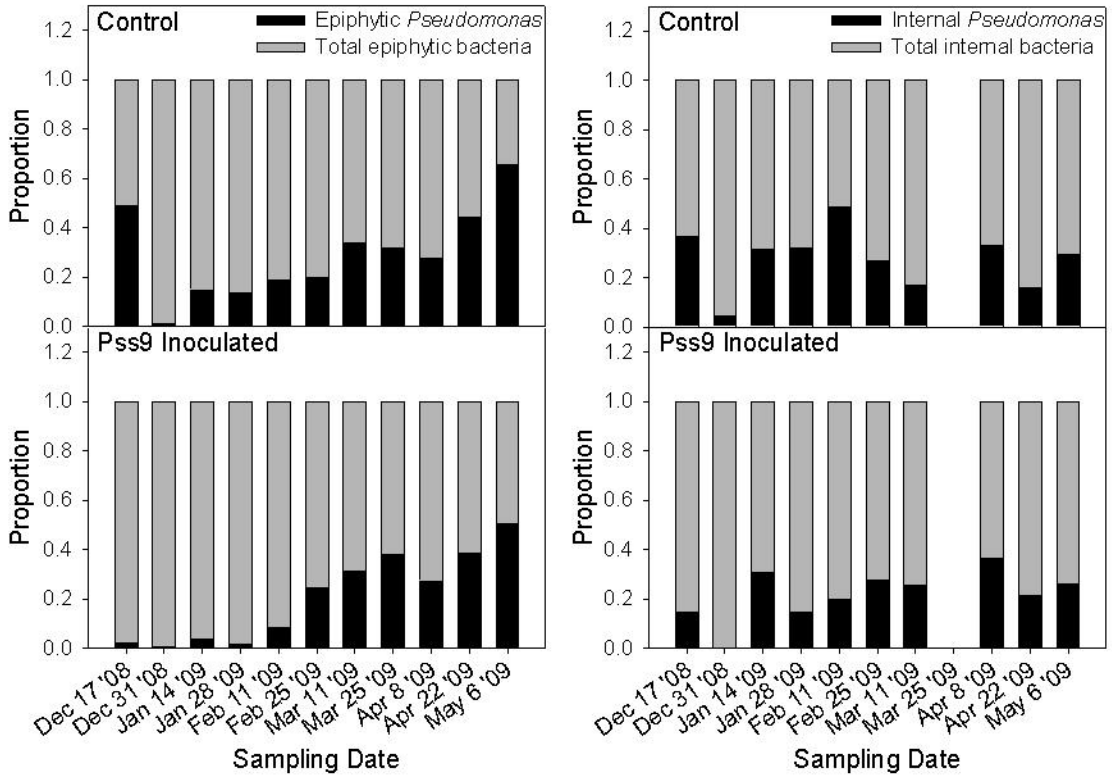

Figure 3-4. Pseudomonads as a proportion of total epiphytic or internal populations recovered from control and Pss9-inoculated blueberry stems for the duration of year 1 of the field trial.

Epiphytic populations (left) and internal populations (right) panels were isolated from control (top) and Pss9-inoculated (bottom) blueberries. Data for internal populations are missing for March 25, 2009.

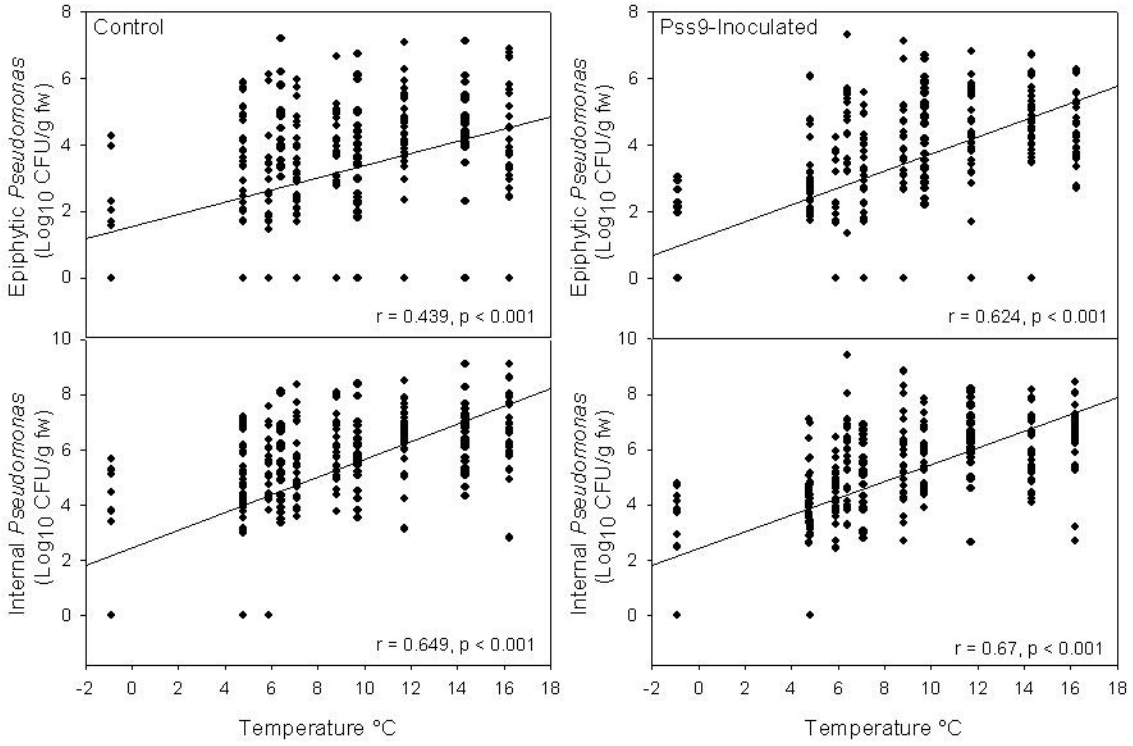

Figure 3-5. Correlation of 14-day average high temperatures with recovered epiphytic and internal* Pseudomonas *from control and Pss9-inoculated blueberry stems in year 1 of the field trial.

Epiphytic (top) and internal (bottom) populations were recovered from control (left) and inoculated (right) blueberries. *R* values represent Pearson's product moment correlation coefficient.

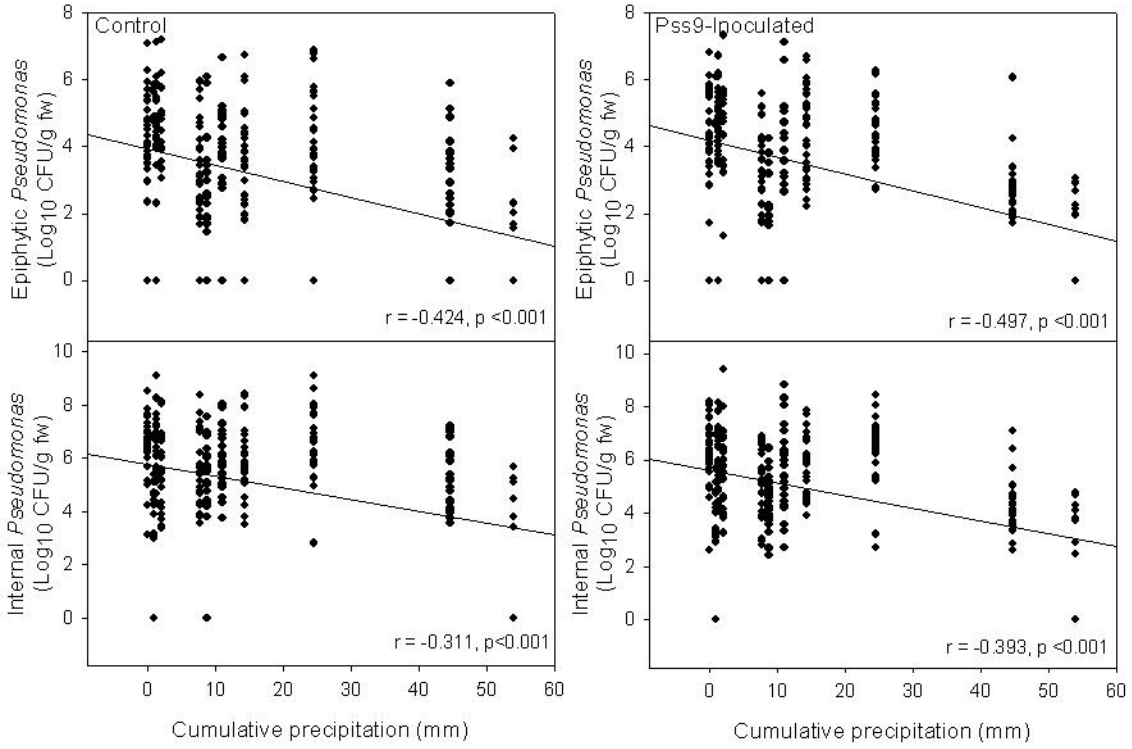

Figure 3-6. Correlation of 4-day cumulative precipitation with recovered epiphytic and internal* Pseudomonas *from control and Pss9-inoculated blueberry stems in year 1 of the field trial.

Epiphytic (top) and internal (bottom) populations were recovered from control (left) and inoculated (right) blueberries. *R* values represent Pearson's product moment correlation coefficient.

Table 3-1. Relatedness of independent correlations between weather variables at four time intervals and recovered epiphytic and internal* Pseudomonas *from control or inoculated blueberry stems in year 1 of the field trial as examined by Fisher's* r- *to-* z *transformations and associated* p *values.

		Epiphytes		Internal	
Weather variable	**Interval**	**|z|**	***p* (two-tailed)[a]**	**|z|**	***p* (two-tailed)[a]**
Average maximum temperatures	14-day	3.43	0.0006	0.46	0.6455
	7-day	3.42	0.0006	0.20	0.8415
	4-day	2.92	0.0035	0.11	0.9124
	2-day	2.79	0.0053	0.14	0.8887
Average minimum temperatures	14-day	3.15	0.0016	0.02	0.9840
	7-day	3.17	0.0015	0.56	0.5755
	4-day	2.85	0.0044	0.78	0.4354
	2-day	2.95	0.0032	0.86	0.3898
Cumulative precipitation	14-day	0.33	0.7414	1.29	0.1971
	7-day	0.45	0.6527	1.19	0.2340
	4-day	1.09	0.2757	1.15	0.2501
	2-day	0.08	0.9362	0.14	0.8887

[a] *p* values > 0.05 are not significant, indicating that correlations for control and inoculated populations are equal, i.e. these populations do not differ in their response to a given weather variable controls and inoculated plants.

significantly different when epiphytic bacteria were recovered from controls vs. Pss9-inoculated plants ($p \leq 0.004$, two-tailed, Table 3-1). No differences in correlations between controls and inoculated plants were seen when internal populations were considered nor when precipitation was the weather variable. Pseudomonads were recoverable throughout the field trial despite the unusually cold and snowy period in December, 2008 (appended Fig. A3-1), indicating that these bacteria are well-adapted to survive under these conditions. Fewest bacteria were recovered at this time but epiphytic populations were still detectable (Figs. 3-1, 3-3).

At the end of the field trial, each plant was examined for disease symptoms. Some winter damage and other lesions were found but very few resembled bacterial blight. All tissue that appeared abnormal was collected and bacteria were extracted. No significant differences were found between numbers of recovered bacteria for controls, Pss9-inoculated, and Pss9R-inoculated when plated on KBC (data not shown). *Botrytis* and

other unidentified fungi were commonly found associated with the samples and more bacteria were recovered on NA than on KBC for all treatments (data not shown).

There were no differences between cultivars in the number of recovered bacteria either epiphytically or internally for any treatment (data not shown). The number of buds associated with twigs macerated for bacterial detection had no correlation to numbers of recovered *Pseudomonas* (data not shown).

Epiphytes were a relatively minor fraction of total recoverable *Pseudomonas* on blueberries in the first year of the field trial. Accordingly, only internal populations were examined in the second year. Introduction of the wounding treatment affected the recovery of *Pseudomonas* from blueberry stems. Wounded-inoculated plants consistently had the highest populations of *Pseudomonas*, ranging from 10^6 – 10^7 CFU/g (Fig. 3-7). Significantly more *Pseudomonas* was found on wounded-inoculated plants than on inoculated plants at all sampling dates ($p \leq 0.01$) except March, 2010. In comparison, *Pseudomonas* populations from wounded control plants differed from non-wounded controls significantly only in the early stages of the field trial (November, 2009 to January, 2010; $p \leq 0.01$). Bacterial populations recovered from wounded-inoculated plants were significantly higher ($p \leq 0.002$) than those on wounded-control plants at sampling times in December, January, April, and May.

Re-isolation of Pss9R occurred more frequently and in higher numbers in year 2 compared to year 1 and was especially prominent on wounded-inoculated plants (Fig. 3-8). Pss9R ranged from a high of $10^{6.7}$ CFU/g in November to $10^{1.2}$ CFU/g in April but maintained relatively high internal populations throughout the trial. Some rifampicin-resistant colonies were recovered from both wounded and unwounded control plants from January-March and in May, 2010.

All wounds remained visible throughout the field trial and many became blackened (necrotic) while others exhibited normal healing (Fig. 3-9). Necrotic wounds were often, but not exclusively, associated with inoculation. When present, necrosis was focused at the incision sites and had minimal outward expansion into adjacent tissue. Blackened lesions never coalesced. In contrast, healed wounds appeared desiccated at the incision site and were surrounded by a red halo (Fig. 3-9B). Secondary infections such

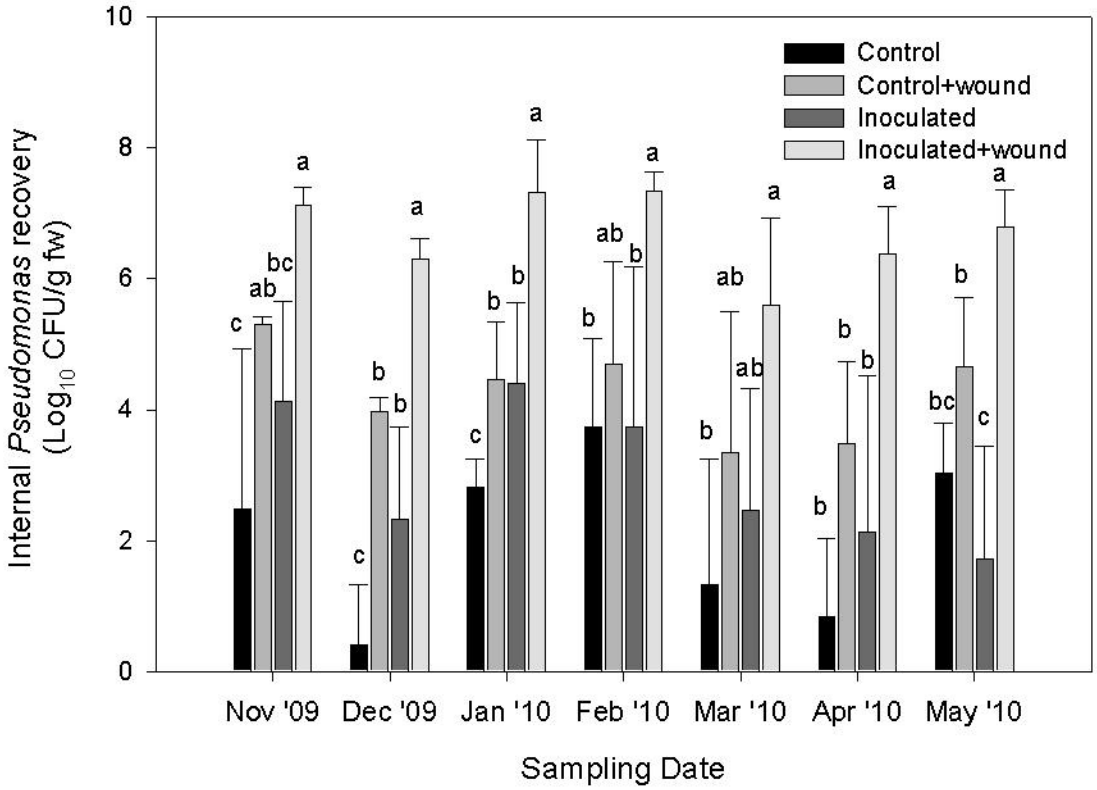

Figure 3-7. Recovery of internal* Pseudomonas *from blueberry stems under four different treatments for the duration of year 2 of the field trial.

Bars with the same letter are not significantly different within a sampling date (α = 0.05). Error bars represent standard deviation (n = 5). Inoculated plants were treated with a mixture of Pss9 and Pss9R inocula.

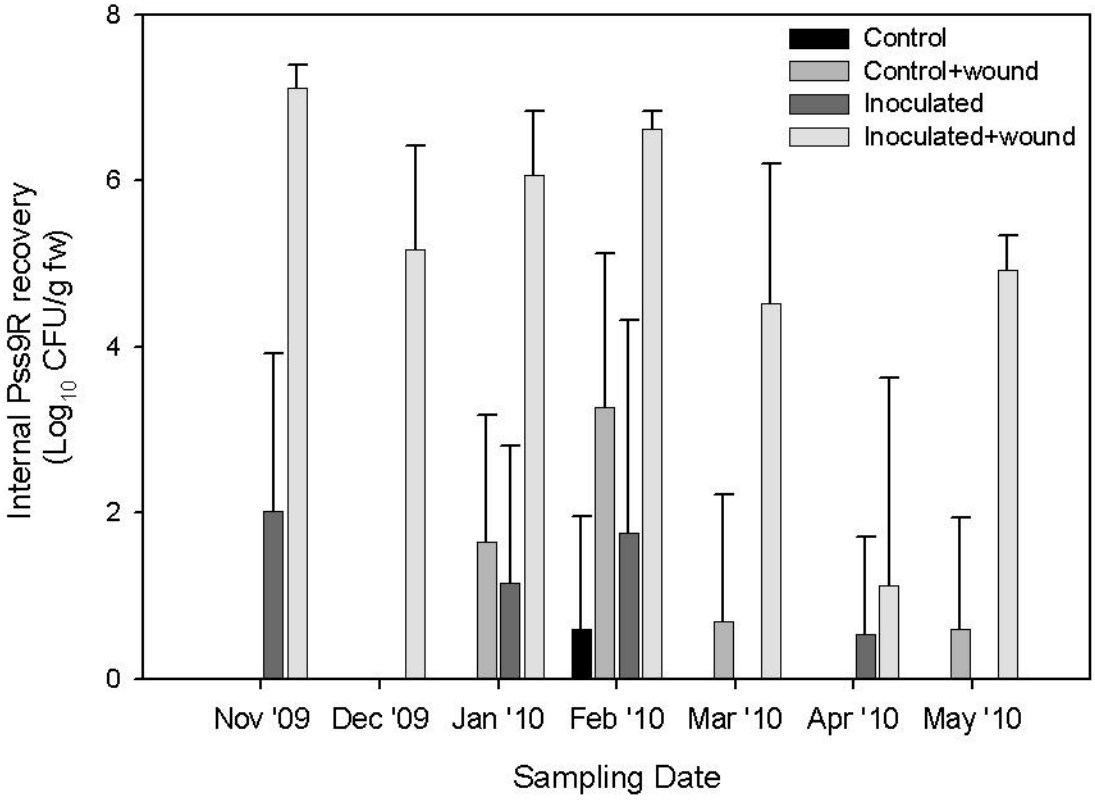

Figure 3-8. Recovery of internalized Pss9R populations from blueberry stems under four different treatments for the duration of year 2 of the field trial.

Error bars represent standard deviation (n = 5).

Figure 3-9. Effect of wounding on blueberry stems in the field.

(A) Wound-inoculated plants frequently had necrotic lesions form at the wound sites but lesions did not expand. (B) Wounded control plants displayed normal healing at the wound site. (C) Blackening of wound sites was apparent one week after the first application of inoculum. (D-E) Wounds remained visible the following spring at (D) bud break and (E) leaf expansion. (F-G) Tip necrosis unrelated to bacterial blight sometimes occurred in relation to wounding. (F) *Phomopsis* lesion on wounded tip. (G) *Botrytis* lesion on wounded tip.

as *Botrytis* sometimes subsumed the wounded tissue (Fig. 3-9G).

Wounding was a significant factor in *Pseudomonas* recovery for the duration of the field trial whereas the inoculation effect was significant only for the early stages of the trial from November, 2009 to January, 2010 (Table 3-2). However, the effects of wounding and inoculation and the wounding *x* inoculation interaction were significant in recovery of Pss9R. For recovery of this strain over the duration of the trial, both wounding and inoculation contributed significantly ($p<0.001$, <0.001). Examination on a month-by-month basis revealed that wounding contributed significantly ($p\leq0.015$) to Pss9R recovery except in November and April. Inoculation was significant ($p\leq0.04$) except from February – April. During this period, detectable numbers of Pss9R had started to spread

Table 3-2. Significance of inoculation and wounding treatments and their interaction in the recovery of Pseudomonas, Pss9R, or total bacteria for each sampling date in year 2 of the field trial.

		Sampling period[a]						
	Treatment[b]	**Nov '09**	**Dec '09**	**Jan '10**	**Feb '10**	**Mar '10**	**Apr '10**	**May '10**
Total Pseudomonads	I	7.18 (0.016)	21.07 (<0.001)	32.50 (<0.001)	3.40 (0.084)	4.22 (0.057)	9.79 (0.007)	0.71 (0.410)
	W	19.94 (<0.001)	83.52 (<0.001)	33.08 (<0.001)	10.21 (0.006)	9.75 (0.007)	25.87 (<0.001)	44.90 (<0.001)
	I*W	0.02 (0.904)	0.19 (0.0670)	2.71 (0.119)	3.40 (0.080)	0.48 (0.499)	1.44 (0.247)	11.91 (0.003)
Pss9R	I	105.02 (<0.001)	82.53 (<0.001)	27.61 (<0.001)	8.36 (0.010)	14.18 (0.002)	1.78 (0.201)	46.46 (0.003)
	W	30.53 (<0.001)	82.53 (<0.001)	38.40 (<0.001)	24.13 (<0.001)	26.07 (<0.001)	0.22 (0.640)	76.01 (<0.001)
	I*W	30.53 (<0.001)	82.53 (<0.001)	9.42 (0.007)	2.02 (0.175)	14.18 (0.002)	0.22 (0.640)	46.46 (<0.001)
Total culturable bacteria	I	6.39 (0.022)	0.09 (0.769)	1.57 (0.228)	2.40 (0.141)	0.01 (0.963)	75.10 (<0.001)	46.24 (<0.001)
	W	10.22 (0.006)	20.93 (<0.001)	20.64 (<0.001)	29.65 (<0.001)	12.27 (0.003)	118.68 (<0.001)	44.51 (<0.001)
	I*W	2.21 (0.157)	0.85 (0.372)	0.49 (0.495)	1.09 (0.311)	0.68 (0.421)	48.03 (<0.001)	39.52 (<0.001)

[a] *F* values for 1 way ANOVA, *p* values in parentheses

[b] I = inoculation treatment, W = wounding treatment, I*W = interaction of treatments

to control plants.

Total culturable bacteria from blueberry stems reached high populations of $10^{9.7}$ CFU/g in April on wounded-inoculated plants (Fig. 3-10). Wounded-control plants had high populations of $10^{7.6}$ CFU/g in February. In the absence of wounding, maximum populations reached $10^{6.6}$ and $10^{6.8}$ CFU/g on controls and inoculated plants respectively. Fewest CFU/g generally were found in December ($10^{3.1}$ on controls, 10^{2} on inoculated and $10^{6.9}$ on wounded-inoculated) except for wounded-controls which had $10^{6.2}$ CFU/g in November.

The proportion of *Pseudomonas* included in total bacteria varied between treatments (Fig. 3-11). Pseudomonads comprised the largest fraction of total bacteria in the wounded-inoculated plants and maintained high levels throughout the sampling period. Pss9R contributed greatly to the proportion of *Pseudomonas* on wounded- inoculated plants and a surprisingly large proportion (approximately 40%) of total bacteria recovered from wounded-control plants in February (Fig. 3-11). Wounded-controls also had high proportions of *Pseudomonas* for the duration of the trial. Pseudomonads on inoculated plants without wounding showed a gradual decline in numbers over the trial period.

Correlations with weather variables did not follow the same pattern seen in the first year of the field trial. No trends were observed between *Pseudomonas* populations and 14-day average maximum temperatures or 4-day cumulative precipitation when examined over the duration of year 2 of the field trial (data not shown). Two treatments, control and wounded-control, did have correlations with 4-day cumulative precipitation but they were moderately positive instead of negative (data not shown). Inoculated treatments also had moderate positive correlations with 2-day cumulative precipitation (data not shown).

When the field trial was considered in terms of seasonal changes and split accordingly into early (winter) and late (spring) phases, many moderate correlations with weather emerged for the first phase (November-January) but completely disappeared for the second phase (February-May, Table 3-3). For the first phase, *Pseudomonas* counts from all treatments had moderate positive correlations with 7- and 2-day average low

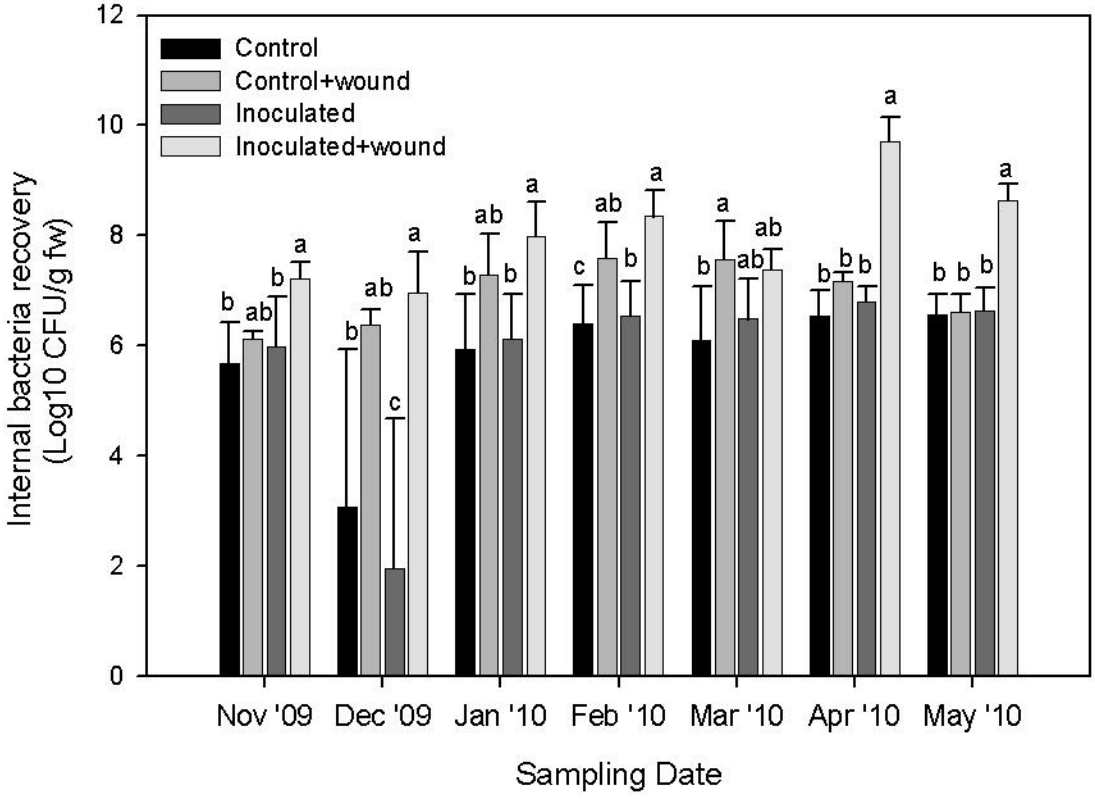

Figure 3-10. Total culturable internal bacteria recovered from blueberry stems during year 2 of the field trial.

Bars with the same letter are not significantly different within each sampling date (α = 0.05). Error bar represents standard deviation (n = 5).

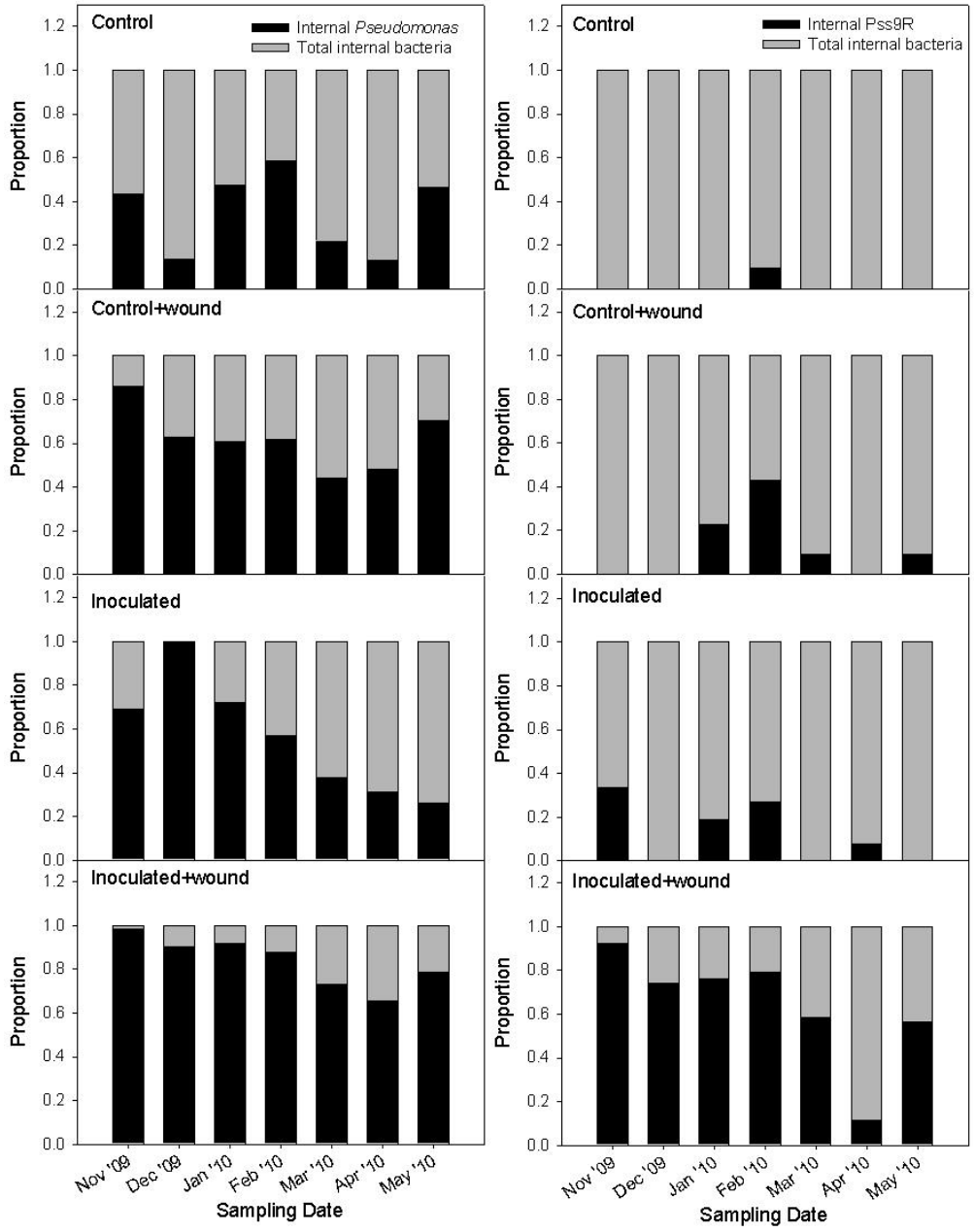

Figure 3-11. **Pseudomonas *and Pss9R as proportions of total recovered internal bacteria from blueberry stems under four treatments over the duration of year 2 of the field trial.***

Total *Pseudomonas* is represented in the left panels and Pss9R is represented in the right panels.

Table 3-3. Relatedness of independent correlations between weather variables and recovered internal* Pseudomonas *from four treatments of blueberry stems in two phases of year 2 of the field trial as examined by Fisher's* r- *to-* z *transformations and associated* p *values.

Phase	Weather variable	Interval (days)	Comparison[ab] C-I |z|	C-I p	CW-IW |z|	CW-IW p	C-CW |z|	C-CW p	I-IW |z|	I-IW p
November-May	Average maximum temperature	14	1.37	0.171	0.77	0.441	0.37	0.711	0.23	0.818
		7	1.43	0.153	0.57	0.569	1.02	0.308	0.15	0.881
		4	1.40	0.162	0.34	0.734	1.21	0.226	0.14	0.889
		2	1.39	0.165	0.18	0.857	1.18	0.238	0.05	0.960
	Average minimum temperature	14	1.84	0.067	0.58	0.562	0.49	0.624	0.49	0.624
		7	1.96	0.050	0.49	0.624	1.76	0.078	0.69	0.490
		4	1.88	0.060	0.72	0.472	1.69	0.091	1.19	0.234
		2	1.28	0.201	0.67	0.503	1.40	0.162	0.55	0.582
	Cumulative precipitation	14	1.15	0.250	0.13	0.897	0.79	0.430	0.37	0.711
		7	0.48	0.631	0.91	0.363	0.50	0.617	0.90	0.368
		4	1.83	0.067	0.04	0.968	0.64	0.522	1.16	0.246
		2	0	1.0	0.29	0.772	0.39	0.697	0.10	0.920
November-January	Average maximum temperature	14	0.01	0.992	1.64	0.101	1.56	0.012	0.13	0.897
		7	0.04	0.968	0.89	0.374	0.93	0.352	0.06	0.952
		4	0.05	0.960	0.63	0.529	0.70	0.484	0.10	0.460
		2	0.04	0.968	0.17	0.865	0.92	0.358	0.06	0.952
	Average minimum temperature	14	1.05	0.294	1.65	0.099	1.57	0.116	0.13	0.897
		7	0.08	0.936	0.64	0.522	0.43	0.667	0.30	0.764
		4	0.08	0.936	0.73	0.465	0.52	0.603	0.31	0.757
		2	0.07	0.944	0.03	0.976	0	1.0	0.24	0.810
	Cumulative precipitation	14	0.08	0.936	1.04	0.298	0.84	0.401	0.30	0.764
		7	0.08	0.936	0.70	0.484	0.49	0.624	0.30	0.764
		4	0.05	0.960	0.35	0.726	0.46	0.646	0.15	0.881
		2	0.07	0.944	0.03	0.976	0.12	0.905	0.22	0.826

[a] Treatment code as follows: C = control, CW = control with wounding, I = inoculated, IW = inoculated with wounding.

[b] Comparisons between pairs of treatments.

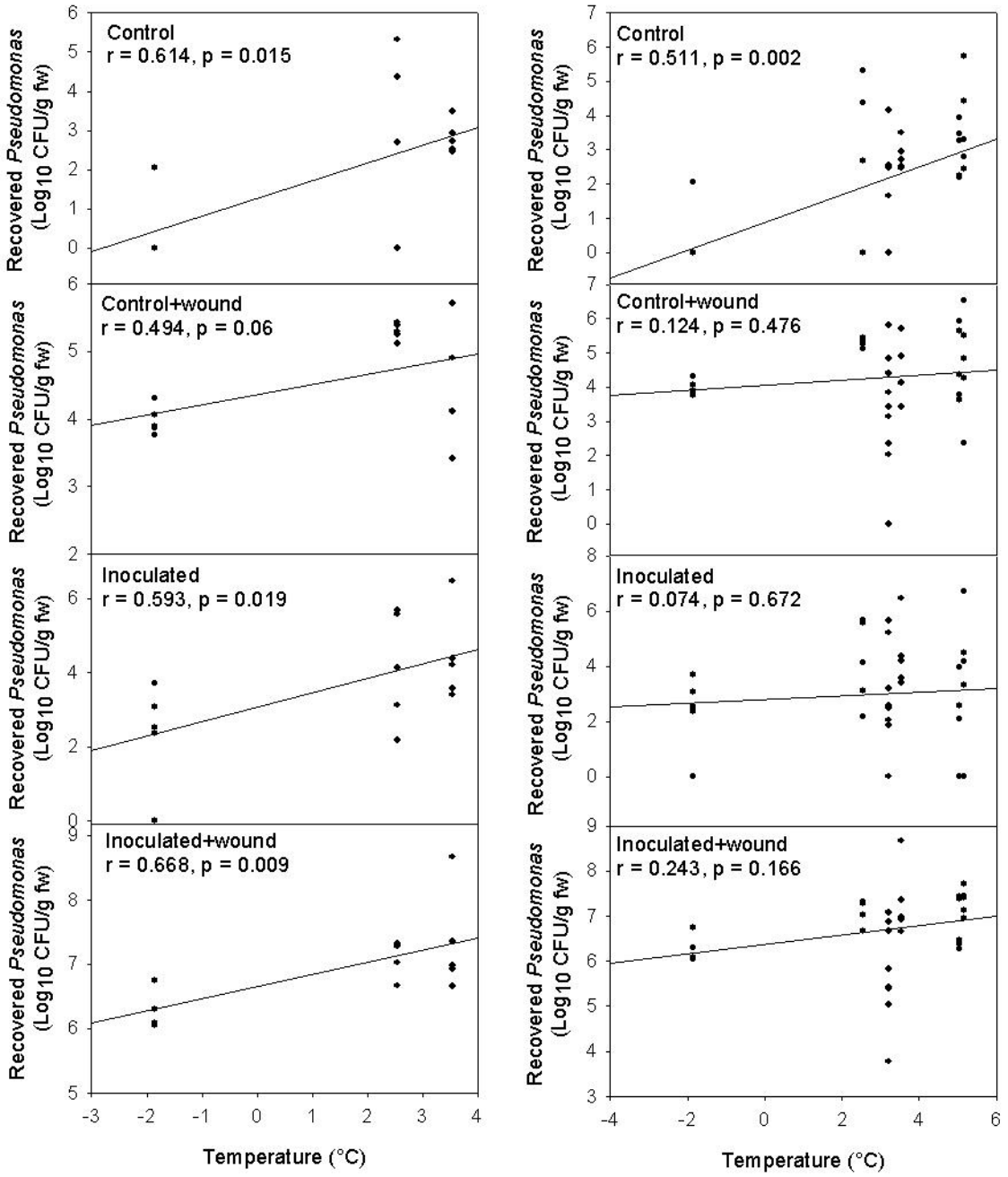

Figure 3-12. Correlation of 7-day average low temperatures with recovered internal* Pseudomonas *from blueberry stems under four treatments in year 2 of the field trial.

Panels on the left represent data for the November-January phase. Panels on the right represent data for the entire duration of the field trial (November-May). *r* values represent Pearson's product moment correlation coefficient.

temperatures (Fig. 3-12), 7-, 4-, and 2-day average low temperatures, and 14-, 7-, 4-, and 2-day cumulative precipitation averages. Independent correlations of weather variables and bacterial populations recovered from different treatments were not significantly different either across all sampling dates or across the November-January phase (Table 3-3).

The importance of weather in dissemination of Pss was demonstrated by the detection of Pss9R on control plants, beginning in January. From the date of inoculation to detection of Pss9R, there was an average of 10.5 hours of wind-driven rain per week at windspeeds greater than 20 km/h including 6 periods of gusting winds at speeds ≥ 50 km/h (appended Fig. A3-3). Treated plants were arranged on the ground cover so that they would be down-wind of prevailing northerly winds for the winter; however, from October to early December wind direction shifted arbitrarily, often coming from the south. This was sufficient to convey Pss9R the 5 m distance between blocks.

Other than necrosis of wounds, no disease symptoms matching those of bacterial blight were noted at the end of the field trial. Winter damage and Botrytis lesions affected the tips of some blueberry twigs.

3.3.2. Hybridization probe results

The probe developed to distinguish Pss from other bacteria and pathovars of *Pseudomonas syringae* was successful. Hybridization was specific to Pss colonies even when other pathovars or bacteria were present on the same membrane (Figs. 3-13, 3-14). During the test phase, the probe hybridized to 4 *P. syringae* pathovars identified by Biolog as *aceris* (isolate 1), *coronafaciens* (isolates 27 and 96), and *lachrymans* (isolate 74). These 4 isolates were then re-tested by PCR amplification of the full-length *syrB* sequence with the B1/B2 primer pair and were reclassified as Pss. The probe never annealed to isolates of *P. viridiflava*, a bacterium commonly associated with blighted blueberry twig samples and capable of growing on KBC (see Chapter 2).

For plated field macerate, the percentage of hybridization-positive colonies was highly variable within treatments and sampling dates (Table 3-4). At all sampling dates except December, the highest number of hybridization-positive colonies was always from inoculated-wounded plants. In December and January, there were no significant

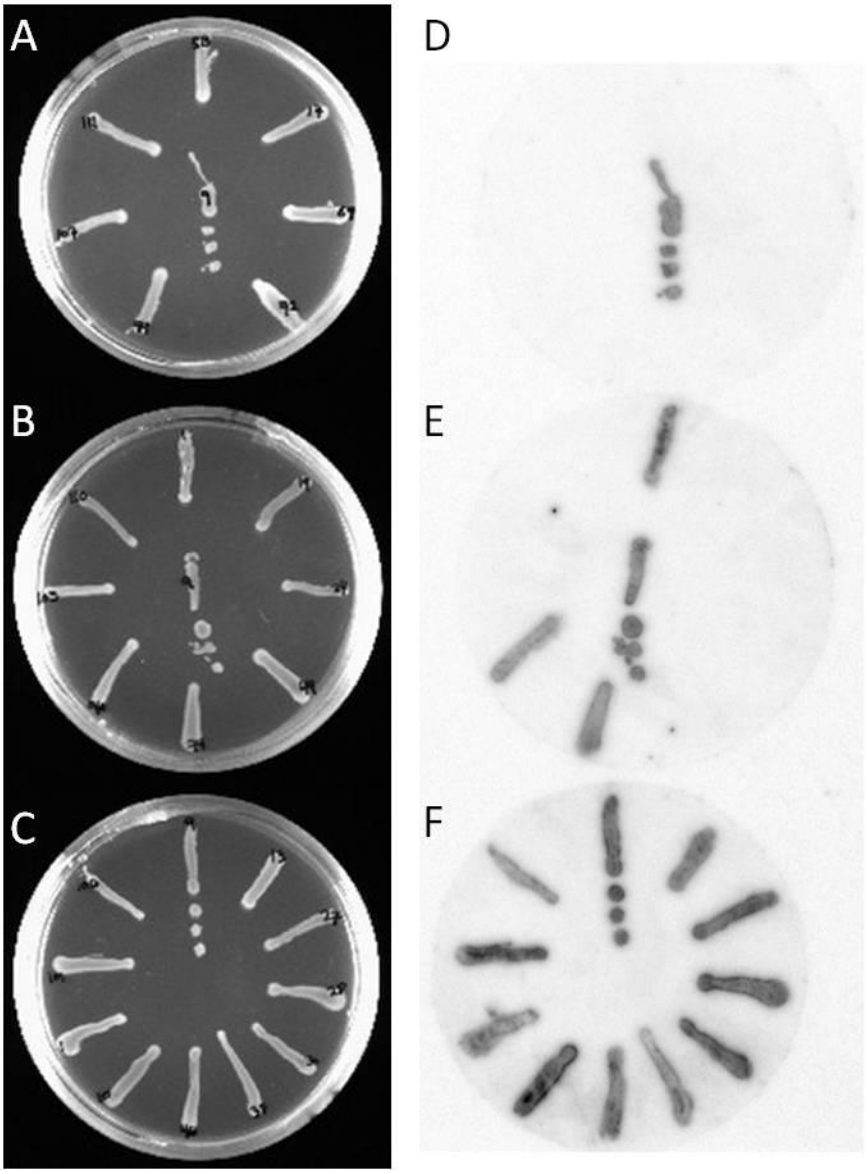

Figure 3-13. Southern dot blot assay with an assortment of* Pseudomonas *field isolates.

(A-C) Selected isolates were applied to agar plates in non-random patterns with Pss9 plated as a streak and 3 dots for a positive control. (D-F) Resulting membranes display positive hybridizations for some isolates. (A) Seven isolates identified as *P. viridiflava* plus Pss9. (B) Eight isolates identified as non-*syringae* pathovars of *Pseudomonas syringae*, *P fluorescens*, and *P. tolaasii* plus Pss9. (C) Ten isolates identified as Pss plus Pss9. (D) No isolates of *P. viridiflava* hybridize with the probe. (E) Three isolates identified by Biolog as *P. syringae* pvs *aceris*, *lachrymans*, and *coronafaciens* hybridize with the probe. These isolates were later confirmed as Pss by PCR. Hybridization-negative isolates were pvs. *apii*, *antirrhini*, and *tagetis* as well as *P. fluorescens* and *P. tolaasii*. (F) All isolates identified as Pss by Biolog or PCR hybridize with the probe.

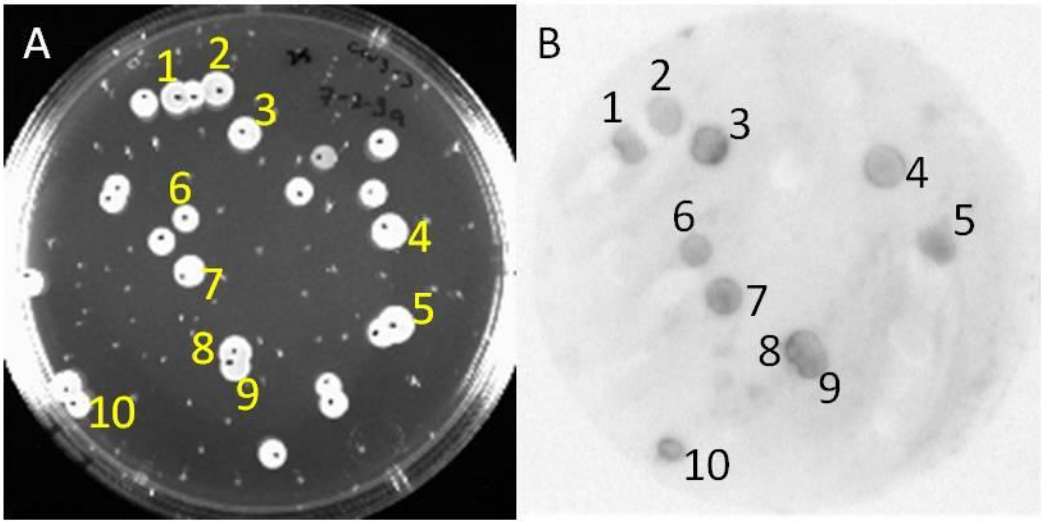

Figure 3-14. Example of selective hybridization with colonies recovered from macerated tissue from the field trial.

(A) Colonies on KBC after 5 days incubation. (B) Ten out of twenty-five colonies hybridized with the Pss-selective probe.

Table 3-4. Percent of plated CFUs that positively hybridize with a probe for Pss by date of sampling.

Sample date	Treatment[ab]							
	C[c]		CW		I		IW	
	% ± SD		% ± SD		% ± SD		% ± SD	
November	nt		30.8±28.7	a	60.4±33.1	ab	70.1±18.6	b
December	nt		3.9±4.4	a	0	a	.7±1.2	a
January	nt		21.0±16.1	a	14.7±13.1	a	31.0±18.9	a
February	39.9±17.2	a	20.7±30.2	a	43.4±21.3	a	80.7±17.9	b
March	14.5±18.3	a	43.7±6.2	ab	67.2±13.4	b	78.2±4.5	b
April	nt		3.3±3.5	a	62.1±50.5	b	76.7±17.2	b
May	27.7±37.5	a	31.1±39.4	a	2.7±3.8	a	74.3±11.3	b

[a] Treatment codes are as follows: C = control, CW = control with wounding, I = inoculated, IW = inoculated with wounding.

[b] Percentage values followed by the same letter are not significantly different for that row.

[c] nt = not tested

differences between any of the treatments. Pairwise comparisons of inoculated-wounded with control-wounded percent positive hybridizations detected significant differences in November (p=0.005), February (p<0.001), April (p<0.001) and May (p=0.013). Pairwise comparisons between inoculated and control plants with no wounding found a significant difference only in May (p=0.039), but there was insufficient data to conduct comparisons with control in November and April. Colonies that grew from macerated field tissues plated on rifampicin-amended medium were 100% positive in hybridization tests, suggesting that all bacteria recovered on KBC+R were re-isolations of Pss9R (data not shown).

3.4. Discussion

Blueberry stems were found to be highly colonized by naturally occurring populations of *Pseudomonas*, even in the absence of disease symptoms. For example, mean epiphytic *Pseudomonas* peaked at (approx) $10^{4.5}$ CFU/g in mid-March and mean internal populations reached $10^{6.5}$ CFU/g by the final sampling of control plants in May of the first year of the field trial. At the same time, inoculation had no effect on numbers of

Pseudomonas recovered. Data from the marked strain Pss9R showed that internal populations were able to establish in a few plants. It is not known why these particular plants supported survival of Pss9R while the majority did not, especially as all plants treated with Pss9R were shown to support large numbers of total bacteria (Fig. 3-3), many of which were *Pseudomonas* (Fig. 3-4). Active defenses involved in limiting bacterial growth would likely not be available during plant dormancy.Rifampicin acts by binding to the β subunit of DNA-dependent RNA polymerase, thereby inhibiting transcription (Campbell *et al*., 2001; Hartmann *et al*., 1985). This affects bacterial growth and metabolism and may account in part for the low recovery of Pss9R from field samples that were already colonized by microbial competitors.

The number of buds on a stem did not correlate with the number of bacteria isolated, suggesting that buds are not an important source of inoculum for blueberry bacterial blight. This is in contrast with other woody hosts such as apple and pear where buds serve as an important site for overwintering (Burr and Katz, 1984; Mansvelt and Hattingh, 1987, 1989) and initiation of disease as blossom infections progress into stems (Hattingh *et al.*, 1989).

Wounding influenced the number of recoverable bacteria on blueberry stems. Greater levels of Pss9R were established on plants in the second year of the field trial in spite of pre-colonization by apparently large endemic *Pseudomonas* populations, which also increased when wounds were present on control plants. Previous inoculation studies that used wounding during the fall season have found it an efficient way to initiate cankers in stone fruit trees (Chandler and Daniell, 1976; Endert and Ritchie, 1984; Perlasca, 1960). No wounding studies have previously been conducted with blueberry plants.

Recovery of Pss9R as an epiphyte at 12 and 22 weeks post-inoculation in the first year of the field trial suggested that internal populations contribute cells to epiphytic aggregates of bacteria, especially as internal populations were found to fluctuate less and may serve as a source of resident populations for blueberry epiphytes. This has been proposed for *P. syringae* on maple (Malvick and Moore, 1988a) and peach (Endert and Ritchie, 1988a) trees. Egress of internalized phytopathogenic bacteria through leaf stomata has been demonstrated for *Xanthomonas axonopodis* pv. *citri* on citrus

(Graham *et al*., 2004), *X. campestris* pv. *pruni* on peach (Miles *et al*., 1977), *P. s.* pv. *morsprunorum* on stone fruit (Roos and Hattingh, 1983), and Pss on asymptomatic apple leaves (Mansvelt and Hattingh, 1989). Similarly, natural openings in stem tissues may serve as exit points for bacteria. For example, *Erwinia amylovora* can leak directly from cankered stem tissue, producing visible exudates originating from lenticels of apple (Eden-Green and Billing, 1972) and pear (Keil and van der Zwet, 1972).

Spread of Pss9R was detected 3 months following application of inoculum during the second year of the field trial. This was probably through wind-driven rain which occurred frequently throughout the trial (appended Fig. A3-3). Simulations of wind-driven rain have demonstrated this to be an effective dispersal method for *Xanthomonas axonopodis* pv. *citri* (Bock, 2005) and *P. s*. pv. *tomato* (Pietarelli, 2006) in the field. Although it was not investigated at the time, spread also might have occurred in the first year of the field trial since plants were not separated by sufficient distance to prevent cross-contamination. Pss was reported to cover distances of 6 m in one day in bean fields (Upper *et al*., 2003). To prevent cross-contamination in future field trials, physical barriers should be erected to separate treatments.

Both vertical and horizontal dispersal of bacteria through the canopy occur (Pietarelli *et al*., 2006); however, vertical dispersal is more common (Constantinidou *et al*., 1990; Malvick and Moore, 1988) and has been detected in fruit orchards (Hattingh *et al*., 1989; Lindow, 1996; Malvick and Moore, 1988, 1988a). Dispersal patterns are predictable (Knudsen, 1989). Rain removes bacterial cells from leaves, largely sending them downwards into the soil (Butterworth and McCartney, 1991) and reductions in leaf-borne Pss populations have been previously reported after rain events (Hirano *et al*., 1994). The role of precipitation for stem-borne bacteria is likely similar. Evidence of this was found in the first year of the field trial when cumulative precipitation negatively correlated with number of *Pseudomonas* recovered as epiphytes and internal populations. It was not clear whether rainfall triggers emergence of internal bacteria prior to washing them off the host. Furthermore, this relationship was not found in the second year of the trial.

Pseudomonad populations varied seasonally on blueberry stems. Although populations seemed to crash in mid-late December, coincident with the coldest temperatures of the year, recovery was seen as rapidly as the following month. Epiphytes seemed to be

more vulnerable to these fluctuations. Immigration may contribute to epiphytic population increases (Hirano *et al*., 1982, 1994), but surrounding grasses, weeds, and other perennial species were not examined as potential reservoirs of Pss. However, epiphytic and internal populations on individual plants may be a single population with ingression and egression (Beattie and Lindow, 1999).

P. syringae may be deposited on plant surfaces as aerosols from the atmosphere (Lindow *et al*., 1988) and in rain or snow (Morris *et al*., 2008) but samples of precipitation were not taken. Susceptibility of peach trees to bacterial canker was greatest during dormancy (Davis and English, 1968; Endert and Ritchie, 1984) although *P. syringae* could be recovered from asymptomatic twigs, scaffold limbs and trunks year-round except in summer in the southern US (Dowler and Weaver, 1975). Cankers on peach trees were produced only by inoculations in February vs. October and when accompanied by wounding (Endert and Ritchie, 1984). Marked Pss strains were found to persist until the following fall (Endert and Ritchie, 1984). It is believed that two periods of infection are involved in bacterial blight on blueberry plants and that they may be distinguished according to the timing of symptom appearance. Infection during the onset of host dormancy in the fall is proposed to produce black-brown stem canker symptoms that appear in late-February to mid-March while infection during release from dormancy in spring is proposed to produce bud blight and tip dieback symptoms appearing in April and May (M.Sweeney, BC Ministry of Agriculture, pers. comm.). It is not known whether separate sources of inoculum are responsible for each infection period but winter damage and spring frosts, respectively, may coincide with these events. These hypotheses could not be confirmed due to the lack of disease development in the field trial; however, it is most likely that blueberry is also most vulnerable to disease during dormancy.

To date there is no evidence that Pss undergoes spontaneous, reversible phenotypic conversions from pathogenic to non-pathogenic forms (phase variation) as demonstrated in *Ralstonia solancearum* (Poussier *et al*., 2003), a phytopathogen able to survive saprophytically in soil. Phase-variable traits such as motility, biofilm production, exo-enzyme production and root colonization have been shown in the rhizosphere pseudomonads *P. putida*, *P*. *aeruginosa*, *P*. *fluorescens*, and *P*. *brassicacearum*

(reviewed in van den Broek *et al*, 2005). The ability to selectively alter phenotype allows for robust responses to environmental stresses.

Foliar disease incidence has been shown to be related to the size of epiphytic populations (Rouse *et al*., 1985). Populations of 10^6 CFU/g are generally conducive to development of disease symptoms (Quesada *et al*., 2010) although as few as 9×10^2 CFU/bud induced pear bud blast (Montesinos *et al*., 1991). Previous reports have shown that symptomless horse chestnut trees harbor internal populations of phytopathogenic *Pseudomonas* (Scortichini and Loretti, 2007); however, these populations were not enumerated. Given the high numbers of Pss recovered from inoculated blueberry stems in this study, the lack of disease typical of bacterial blight must be dependent on other factors. Although wounding did increase colonization by Pss, it did not lead to pronounced development of symptoms. Environmental variables are likely the key to development of this disease in the field.

The high degree of adaptation of Pss to blueberry stems was demonstrated by its ability to survive the shift from the laboratory environment to late season field conditions, including abnormally low temperatures of less than -10°C. These bacteria may have been at a competitive disadvantage against naturally established strains. Studies have shown that batch fermentation in laboratory media can alter their ability to nucleate ice (Blondeaux and Cochet, 1994; Pooley and Brown, 1991), produce effectors (Huynh *et al*., 1989; Kim *et al*., 2009) influence cell size (Roszak and Colwell, 1987; Sezonov *et al*., 2007), and alter nutrient use (Scortichini and Rossi, 1995). Blueberry does not seem to suffer from blight to the same degree as other woody hosts. For example, cherry tree trunks and scaffold limbs are reported to develop cankers that girdle and kill entire trees (Kennelly *et al*., 2007).

Is Pss an accidental pathogen? Under apparently rare circumstances, Pss seems to kill blueberry tissues (tips, buds), perhaps by nucleating ice and causing frost damage that is more extensive than in an average year. Increased ice nucleation may do more harm to woody hosts grown in warmer climate orchards (*e.g*. European hazelnuts) but northern highbush blueberry is a cold-climate species and requires hours of chilling to properly set buds (Eck, 1988; Gough, 1994). Cold-acclimated, dormant blueberry buds have been shown to form ice crystals only in extraorgan spaces as a survival

mechanism (Flinn and Ashworth, 1994). Because highbush blueberry is cold-adapted, ice nucleation may only affect the tender parts of the plant (tips and buds) in particularly frosty years or when frosts occur while the plant is not cold acclimated. Pss is not known to harm blueberries to the extent that it is reported on other orchard trees (*e.g.* pear, peach, cherry) but has set back young plantings in the Fraser Valley by blighting the buds that would generate architectural and fruiting wood. Control of this sporadic disease would be advanced by the development of a local disease forecasting system based on predisposing environmental variables, inoculum load, and current physiological state of the host.

4. General Discussion and Conclusions

From the point of view of a microbe, the topography of a plant is vast and changeable. Abaxial and adaxial surfaces of a single leaf can differ with respect to the presence or absence of stomata and trichomes, frequency of these structures and the degree of exposure to solar irradiation. Gradients of humidity and temperature over the leaf area exist due to variability in the thickness of the boundary layer (a zone of wind resistance and convective heat transfer surrounding each leaf; the zone is thinnest at the windward edge) (Vesala, 1998). The epiphytic lifestyle is challenging and this may explain why the known plant-associated eubacteria are limited to three phyla: Proteobacteria, Firmicutes and Actinobacteria (Beattie, 2006). Some of the best-known plant symbionts (rhizobia) and pathogens (*e*. *g*., *Erwinia*, *Agrobacterium*, xanthomonads, pseudomonads) are circumscribed by a single phylum, Proteobacteria. Findings from this study confirm that *Pseudomonas* is a prominent part of the microbial flora that colonizes blueberry stems. In two consecutive years of field trials, dormant blueberry stems were found to harbour high populations of *P. syringae* and many of the recovered bacteria were confirmed to be Pss by DNA-DNA colony blot hybridizations in the second year. The identity of other bacteria on blueberry remains unknown. The following points are presented for discussion and future research ideas:

Use of the semi-selective KBC medium allowed efficient capture of *Pseudomonas* isolates but consequently prevented gathering information on the complete bacterial communities associated with blighted blueberry tissues. Numbers and types of other phylloplane bacteria on blueberry plants remain unknown but it would be interesting to investigate the composition of the entire microbial assemblage, whether and when other bacteria compete or co-exist in this niche, and possible roles in prevention or enhancement of bacterial blight symptoms. As with the human microbiome (Peterson *et al*., 2009), whole-community analysis of the bacterial populations on plants is now possible. Culture-independent methods have been recommended for examination of the total microbial diversity on plant surfaces (Whipps *et al*., 2008) given the inefficiency and

possible inaccuracy of culturing methods (Roszak and Colwell, 1987). Also, recent studies using non-culture-dependent techniques have revealed a much broader array of bacteria on plant surfaces (Yang *et al*., 2001). Assessing the role of non-cultured microbes in pathogenicity and/or prevention of disease would be difficult. There is also a question of whether testing isolates individually truly reflects the potential pathogenic activity of a community. Consortia of phytopathogenic microbes have not been explored as have, for example, the consortia of nitrifying bacteria.

P. syringae has been described as a repository for all unclassified fluorescent phytopathogenic bacteria (Young, 2010). Taxonomy of bacteria based on phenotype (ability to produce EPSs, pathogenicity on a specific host range, metabolic profiling, etc.) is informative but less precise than the resolution of species and subspecies provided by comparisons of genotypes. The taxonomic status of fluorescent phytopathogenic pseudomonads has been clarified somewhat (Bull *et al*., 2011; Manceau and Horvais, 1997; Young, 1992) but remains problematic. Current bacterial classifications represent the best consensus but are understood to be imperfect.

Phylogenetic groupings within Pss can be based on host range (woody vs. herbaceous plants) and the associated effectors that endow specific pathogenicity on distinct types of plants (Cirvilleri *et al*., 2006; Sarkar *et al*., 2006). For example, isolates from bean, such as Pss B782a, clustered separately from other Pss strains in phylogenetic analyses (O'Brien *et al*., 2011; Sarkar *et al*., 2006). Phenotypic differences such as this ability to cause disease on some hosts and not others suggest underlying genotypic differences which could be used to identify and classify bacteria (Scholz *et al*., 1994). Detection of the *syrB* gene cluster for positive identification of a bacterial isolate as Pss has been recommended (Sorensen *et al*., 1998) and was used in this study; however, this may be insufficient. Two pathovars closely related to pv. *syringae* – *aptata* and *atrofaciens* – were reported to produce syringomycin and derivative toxins using the *syrB*-encoded enzymes (Bultreys and Gheysen, 1999; Vassiliev *et al*., 1996). It has been suggested previously that these pathovars may actually be synonymous with pv. *syringae* (Gardan *et al*., 1994; Sawada *et al*., 1999) but they remain valid taxa to date. No matches were found by BLAST for the Pss9 or B728a *syrB* sequences and the available *aptata* genome (GenBank accession AEAN00000000.1).

Correct identification of the etiologic agent is important for understanding disease. Knowing the pathogen, its lifestyle and its interactions with hosts enables application of the appropriate controls in an opportune manner. Of the many methods available for identifying bacterial isolates, Biolog® is purported to be efficient and accurate (Krejzar *et al*., 2004; Truu *et al*., 1999; Jones *et al*., 1993). Although useful for identifying bacteria to the genus and species levels, it mis-identified the *P*. *syringae* pathovar of interest several times in this study. This inability to distinguish strains at the pathovar level has been noted by other authors (Hofte and de Vos, 2006). Biolog results should be confirmed with other methods, especially if identification to pathovar level is of importance. DNA sequencing and fingerprinting have been proposed as two alternatives and techniques such as rep-PCR (Vicente and Roberts, 2007) and multilocus sequence typing (MLST) (Sarkar and Guttman, 2004) have been demonstrated to work with Pss.

Two isolates of Pss failed to produce symptoms on lilac. This was possibly due to experimental error such as misplacement of the inoculum droplet (i.e., on an unwounded axil) or over-dilution of inoculum, but the possibility that they are non-pathogenic exists. Previous reports have indicated that not all *P*. *syringae* isolates are pathogenic (Mohr *et al*., 2008). Failure of an isolate to produce disease may be the result of a combination of factors, such as growth under laboratory conditions and attenuation through repeated culturing (*cf*. development of BCG vaccine through repeated culturing of *Mycobacterium* on synthetic medium), or the lack of some unknown trigger for virulence. For example, strains of Pss were reported as not producing toxins on laboratory medium which lacked plant signals but did produce toxins when the medium was amended to stimulate this reponse (Mo and Gross 1991, Mo *et al*., 1995, Wang *et al*., 2006). It was assumed that Pss isolates from blueberry would be pathogenic on lilac as reported in the literature (Scheck *et al*., 1998); however, it is possible that this cross-pathogenicity did not hold true for these two isolates. Other isolates of Pss have failed to induce disease on plant species from which they did not originate in spite of the test plant falling within the expected host range (Morris *et al*., 2000). Some authors have argued that this merely reflects the poor taxonomy of the pathovar system (Young, 2010).

The relationship between quantity of Pss9, disease initiation and disease severity was examined through the serial dilution inoculation assay on lilac plantlets. It was expected that disease from small doses of bacteria (10^1-10^2 CFU) would expand in a predictable

manner ending in plantlet death, but disease did not progress past a certain level of severity. It is unknown whythe bacteria failed to multiply and cause disease to an unlimited extent under ideal conditions; namely, a weak and wounded host under warm, humid conditions. Something appears to restrict disease progress when the initial dose is low but whether this is due to a host response, self-attenuation of virulence on the part of the bacteria, or a combination of these is unknown. It would be interesting to enumerate the bacteria throughout a trial to determine whether there truly are fewer bacteria on plantlets with stalled disease progression and less severe symptoms. Previous studies in the field have shown that disease development is linked to amount of inoculum present (Hirano and Upper, 1989, 1990). Predictive modelling of disease severity based on bacterial populations on lilac was not done for this study but it could be a starting point for extrapolating these findings to blueberry plants in the field.

The reaction of blueberry leaf tissue in laboratory pathogenicity assays depended on leaf age, phenological status of the bush from which tissue was harvested and cultivar. Age of host tissues in relation to disease resistance has been referred to as ontogenic resistance, developmental resistance and age-related resistance, or ARR (Develey-Riviere and Galiana, 2007). ARR in trees has been studied in relation to its role in anti-herbivory, especially in seedlings (Bryant and Julkunen-Tiitto, 1995; Webber and Woodrow, 2009). Less is known about its role in disease resistance. One study on growth chamber reared seedlings of cotton, an annual shrub, showed increased terpenoid levels in healthy 12-day old seedlings compared with diseased 5-day old seedlings when each were challenged with the damping-off fungus, *Rhizoctonia solani* (Hunter *et al*., 1978). A study with *Schefflera arboricola* (dwarf umbrella plant, an evergreen shrub commonly used as a houseplant) found evidence of ARR in response to leaf infection with *Pseudomonas cichorii* in greenhouse raised plants (Chase and Jones, 1986). For future testing of blueberry, field-grown tissues should be collected within a short period if results from separate trials are to be combined. Further studies would be needed to clarify the details of this reaction.

Restriction of bacterial proliferation was greater in Elliott leaf discs than in Duke discs. This suggests that Elliott has true resistance to bacterial blight and is not just escaping infections as a late bloomer; therefore, field observations of Elliott's resistance may be valid. Other cultivars that appear resistant to bacterial blight are "June", "Rancocas",

and “Weymouth” (Pscheidt and Ocamb, 2008; Caruso and Ramsdell, 1995; Gough, 1994). Rancocas and Elliott have partially shared heritage through germplasm contributed by “Rubel”, a wild selection introduced into blueberry breeding programs in 1911 (Gough, 1994). Rubel has been shown to have the highest levels of antioxidant compounds in berries among 86 cultivars and Rubel *x* Duke progeny showed negative epistasis (lowering of fitness by allelic interaction) for antioxidant capacity (Ehlenfeldt and Prior, 2000). The role of antioxidants in disease resistance is discussed below. Duke has substantially less germplasm contributed by Rubel than Elliott and Rancocas, however it is not known whether bacterial blight resistance was inherited through other breeding sources. Blueberry breeding programs could make use of this type of leaf assay when selecting parent genotypes for crossing for disease resistance.

Blueberry leaves also have high innate levels of phenolics such as proanthocyanidins (prodelphinidins and procyanidins), flavonols (quercetin and kaempferol) and hydroxycinnamic acids (*p*-coumaric acid and caffeic or ferrulic acids; Riihinen *et al*., 2008). Studies have shown that levels of these compounds vary between blueberry fruits at different maturation stages (Castrejon *et al*., 2008), from different cultivars (Piljac-Zegarac *et al*., 2009; Connor *et al*., 2002; Prior *et al*., 1998) and from different geographic locations (Connor *et al*., 2002) but variation of phenolics within other plant organs is not as well-studied. One exception was a study that showed higher levels of the flavonols quercetin and kaempferol in red leaves (sun-exposed) than green leaves (shaded) of bilberry (*Vaccinium myrtillus*), due to their role in photo-protection (Jaakola *et al*., 2004). The first leaves of the season for different blueberry cultivars have marked differences in colouration. Duke’s first leaves have a very red appearance which proceeds to fade as leaves “green up” while Elliott’s never have a red appearance. One-year old wood also is red in winter and greens up in spring but this happens for all cultivars as chlorophyll is scavenged during preparation for dormancy. Derivatives from *p*-coumaric acid are involved in plant defenses induced by both pathogens and wounding and lignin biosynthesis (Dixon and Paiva, 1995). It is possible that increasing levels of these compounds were also responsible for controlling bacterial proliferation in blueberry leaf discs collected later in the season.

Are toxins necessary for pathogenesis? It may be that toxin-induced necrotic symptoms occur only during unrestricted proliferation of Pss within plant tissues, concurrent with

increased production of toxin leading to excessive leakiness in host cell membranes and ultimately death. Accumulated indirect evidence from this study (detection of syrB gene, expression of same *in planta* and *in vitro* fungistasis by extracts) strongly suggests that Pss9 produces syringomycin (or other CLPs). This could be confirmed in the future by direct detection of toxins via HPLC (Hutchison *et al*., 1995) or SDS-PAGE plus Western blotting optimized for small peptide detection (Zhang *et al*., 1997). Regardless, the pathogenicity of Pss9 was sometimes ambiguous as demonstrated by the failure to kill lilac plantlets with low initial inoculum load, restriction of colonization by mature blueberry leaf tissue and failure to produce disease symptoms in field trials. In agreement with other authors, the importance of syringomycin in the lifestyle of Pss is therefore not restricted to virulence.

Populations of Pss and disease incidence and severity could not be correlated due to the absence of bacterial blight in the field trials. Although introduction of a wounding variable greatly enhanced internal colonization by Pss, this was still insufficient to reproduce symptoms seen in the field in years of severe blight. The reasons for this are unknown but are suspected to be environmental. Other researchers in the PNW have also been unable to induce bacterial blight on blueberry in recent years (M. Sweeney, BC Ministry of Agriculture, pers. comm.).

Pss9R applied as a topical spray in December, 2008 was most frequently recovered as internalized bacteria. This strain was sufficiently competent to gain entry into the plant, survive, and later multiply to detectable levels. Although no wounding variable was introduced at the time of inoculation in the first year of the field trial, sampling involved repeated wounding of plants which may have aided entry of epiphytic cells of Pss9R into stems. Alternatively, Pss9R may have entered through lenticels. Lenticels of blueberry were not examined during the field trial but some tree species are known to form closing layers (a rigid layer of suberized cells formed during the final phellogen growth for the season) for overwintering (Graca and Pereira, 2004; Kalachanis and Psaras, 2007; Rosner and Kartusch, 2003) while other species do not (Kalachanis and Psaras, 2007). Closing layers may prevent entry and spread of bacteria within the airspaces beneath lenticels. Compared to wounds, lenticels often are considered to be secondary routes of entry for bacterial pathogens (Agrios, 1997) but it has been shown that *P. syringae* pv. *aesculi* directly infects European horse chestnut stems through lenticels during the

dormant season (Steele *et al*., 2010) and *P*. *s*. pv. *actinidiae* mainly colonizes kiwi vines through lenticels (Serizawa and Ichikawa, 1994; Scortichini *et al*., 2012). Results from this study support the secondary role of lenticels in bacterial entry since internal populations of Pss9R were much larger in the field trial which included wounding.

Symptomless, wild hazelnut trees were found to harbor internal populations of *P. syringae* with demonstrated pathogenicity in laboratory testing (Scortichini and Loretti, 2007). The role of the potentially pathogenic strain was described as "unknown" in healthy tree tissues. Quesada *et al*. (2010) demonstrated that symptomless stems and leaves from olive trees inoculated with *P*. *savastanoi* pv. *savastanoi* (Psv) carried epiphytic populations of 10-10^4 CFU/g while uninoculated tissues had 0-10 CFU/g. Internal populations were not enumerated at the time; however, a subsequent study (Krid *et al*., 2010) showed that Psv populations within olive knots was 10^6 CFU/g while within asymptomatic leaf tissue, populations reached 10^4 CFU/g. In both studies, epiphytic and internal populations fluctuated seasonally. Pss populations within asymptomatic blueberry stems were found to be up to two orders of magnitude larger: 10^6—10^7 CFU/g without wounding and 10^7—10^8 CFU/g with wounding. It is not known if blueberry is a host with a particularly high carrying capacity for pseudomonads or if local Pss isolates are well-adapted to proliferating within host tissues without causing disease in years with average weather.

There seem to be other differences in the epidemiology of bacterial blight on blueberry compared to other woody hosts as well. Neither floral nor vegetative buds were found to increase the number of bacteria isolated from blueberry stems, suggesting that buds are not an important source of inoculum for bacterial blight unlike in cherry (Roos and Hattingh, 1988; Sundin *et al*., 1988), pear (Mansvelt and Hattingh, 1987, 1987b, Natalini *et al*., 2006) and apple (Burr and Katz, 1984; Leben, 1981; Mansvelt and Hattingh, 1987). However, the current study was not designed to systematically answer this specific question. The importance of buds in blueberry disease could be examined with histological methods to ascertain the preferred sites of colonization on stems. This would be useful since buds are often killed by bacterial blight while stems remain healthy. Buds are considered protected sites for bacteria because they offer more moisture and less UV exposure (Leben, 1981). Does Pss proliferate within buds during periods in spring when the potential for late frosts may assist in killing the tissues?

Blueberry also does not seem to suffer from blight to the same degree that *Prunus* species do. For example, trunks and scaffold limbs of cherry can develop cankers that girdle and kill entire trees and limbs (Kennelly *et al*., 2007). Stem infections in cherry can begin in blighted buds followed by internal dispersal of bacteria (Kennelly *et al*., 2007). This was not typically seen in blueberry; however, sampling was done during years of mild blight. Blueberry and cherry do share a propensity for damage to younger plants rather than older ones. A comparison of the different constitutive defenses, such as phenolics, between these two host species may reveal differences in types and quantities of compounds related to differences in susceptibility to bacterial blight. This would provide breeding targets for enhanced disease resistance.

Cooler temperatures (16°C vs. 28°C) have been shown to induce expression of virulence factors in phytopathogenic bacteria, including *P. syringae* toxins, *hrp*s, ice nucleating proteins and exopolysaccharides (Smirnova *et al*., 2001). However, laboratory studies conducted at temperatures near and below zero Celsius have focused only on ice nucleation (*e*. *g*., Anderson and Ashworth, 1986; Kozloff *et al*., 1983; Lindow, 1988; Maki *et al*., 1974) or growth dynamics (Young *et al*., 1977). The behaviour of plant-associated bacteria at near-zero temperatures in a field setting is not well studied. It has been suggested that environmental conditions modulate phase variation in many clinically important bacteria (Henderson *et al*., 1999). This is thought to enhance bacterial survival by producing mixed populations with members that are always ready to respond appropriately to random environmental changes (Henderson *et al*., 1999). It is not known if Pss uses this strategy to cope with changes in climate variables.

If the dynamics of Pss, blueberry, and weather were better understood, it would be possible to accurately forecast disease. Warning systems have been developed to alert growers when environmental conditions plus inoculum load indicate possible epidemics. Latorre *et al*. (2002) could predict pear blossom blast caused by Pss based on air temperature plus moisture levels during a susceptible blossom stage. These warnings could be passed on to growers who would then take precautions to ameliorate losses. For example, preventative applications of bactericide might reduce damage to plants in the field. Studies using controlled environment chambers would help pinpoint the environmental factors that have the largest impact on development of bacterial blight.

Although temperature and rainfall are probably the most important, the timing of cold and warm weather or rainfall duration might also have an effect.

Field pathologists generally agree that climate change could be the impetus for new crop diseases to flourish. Both warming and cooling trends would alter the profile of pathogens in local fields as each type of climate is preferred by some pathogens over others. Pss and many other phytopathogenic bacteria thrive under cool, wet conditions (Crosse, 1966; Gross *et al*., 1983) while fungal pathogens may prefer either warm or cool temperatures. All of the fungi used in the *in vitro* assays for syringomycin were isolated from blueberry tissues. This is the first report of *Sclerotinia* on blueberry in Canada. Worldwide, *Sclerotinia* has been reported as a blueberry pathogen twice: originally in Japan (Umemoto *et al*., 2007) and subsequently in Argentina (Perez *et al*., 2011). *Sclerotinia* was recovered from blighted blueberry twigs while surveying and isolating for pseudomonads in spring, 2008, from a field adjacent to vegetable farms (a potential inoculum source) in Cloverdale, BC. Fungal identification was by morphology on PDA (white fast-growing mycelium, absence of conidia and production of abundant black, irregularly shaped sclerotia) and PCR amplification and DNA sequencing of the ITS region (100% sequence similarity with accessions). Pathogenicity tests were not conducted at the time of this study; however, *Sclerotinia* may be a new threat to blueberry production in the Fraser Valley. Bacterial blight may also become a disease of greater importance in the future, especially since the environmental triggers for outbreaks are not fully understood. Environmental shifts to longer periods of cool, wet weather in the Fraser Valley could result in inoculum loads of Pss that are unmanageable.

Further exploration of the relationship between pseudomonads and blueberry plants is necessary to fully understand the triggers for disease outbreaks. Pathogens evolve to attenuate virulence, preventing host death and thereby increasing their own fitness (Bull, 1994; Maurelli, 2006). Non-adapted microbes are virulent, but those that co-evolve become mutualists or commensals (Bull, 1994). It would seem that, ordinarily, Pss is a highly adapted and harmless bacterium that colonizes blueberry and, perhaps, prevents other, more virulent microbes from establishing on the plants. Hirano and Upper (1990) argue that Pss on bean follows a similar pattern: the norm for Pss is a non-pathogenic, epiphytic lifestyle. Instances of bacterial leaf blight on bean occur when large

populations “stimulate” the plant to release more nutrients than normal, causing cell death and destruction of habitat (Hirano and Upper, 1990). In such a case, Pss survives at the lesion margins until it can migrate to a new leaf surface. Pss will always have a new blueberry stem or bud to colonize since they are perennial hosts.

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Appendices

Appendix A.

Supplemental figures and tables

A1. In vitro assays of fungal growth inhibition by bacterial extracts and live cultures.

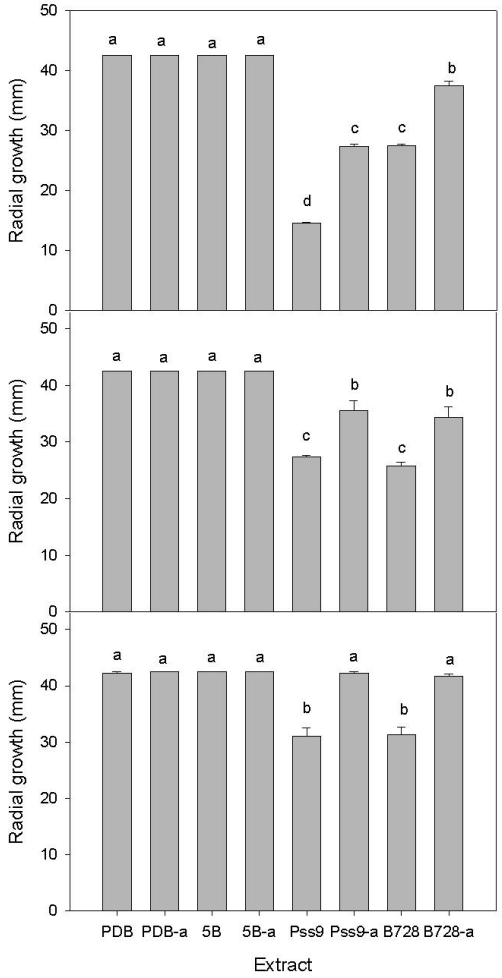

Figure A1-1. In vitro growth inhibition assays of Sclerotinia challenged with crude bacterial extracts and autoclaved extracts.

Top, centre, and bottom panels represent results from three independent trials. PDB = extraction of sterile potato dextrose broth, PDB-a = autoclaved extract of sterile broth, 5B = *P. viridiflava* syringomycin-negative control, 5B-a = autoclaved extract, B728 = Pss syringomycin-positive control, B728-a = autoclaved extract. Fungal plugs and 10 µL droplets of extracts were co-plated on PDA. The zone of inhibition between droplets and mycelia was measured after 3 days incubation. Bars with the same letter are not significantly different for that trial. Error bars represent the standard error of the mean.

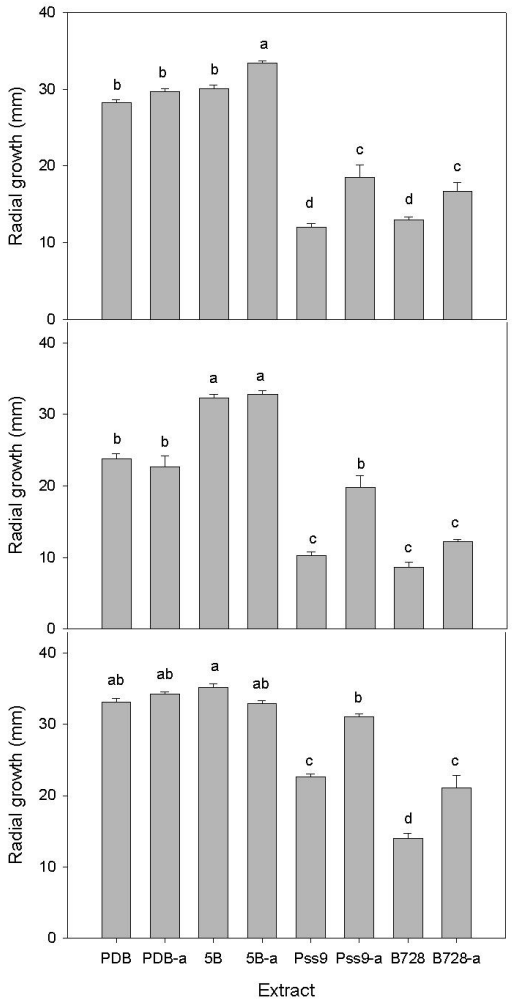

Figure A1-2. In vitro growth inhibition assays of Phomopsis challenged with crude bacterial extracts and autoclaved extracts.

Top, centre, and bottom panels represent results from three independent trials. PDB = extraction of sterile potato dextrose broth, PDB-a = autoclaved extract of sterile broth, 5B = *P. viridiflava* syringomycin-negative control, 5B-a = autoclaved extract, B728 = Pss syringomycin-positive control, B728-a = autoclaved extract. Fungal plugs and 10 µL droplets of extracts were co-plated on PDA. The zone of inhibition between droplets and mycelia was measured after 5 days incubation. Bars with the same letter are not significantly different for that trial. Error bars represent the standard error of the mean.

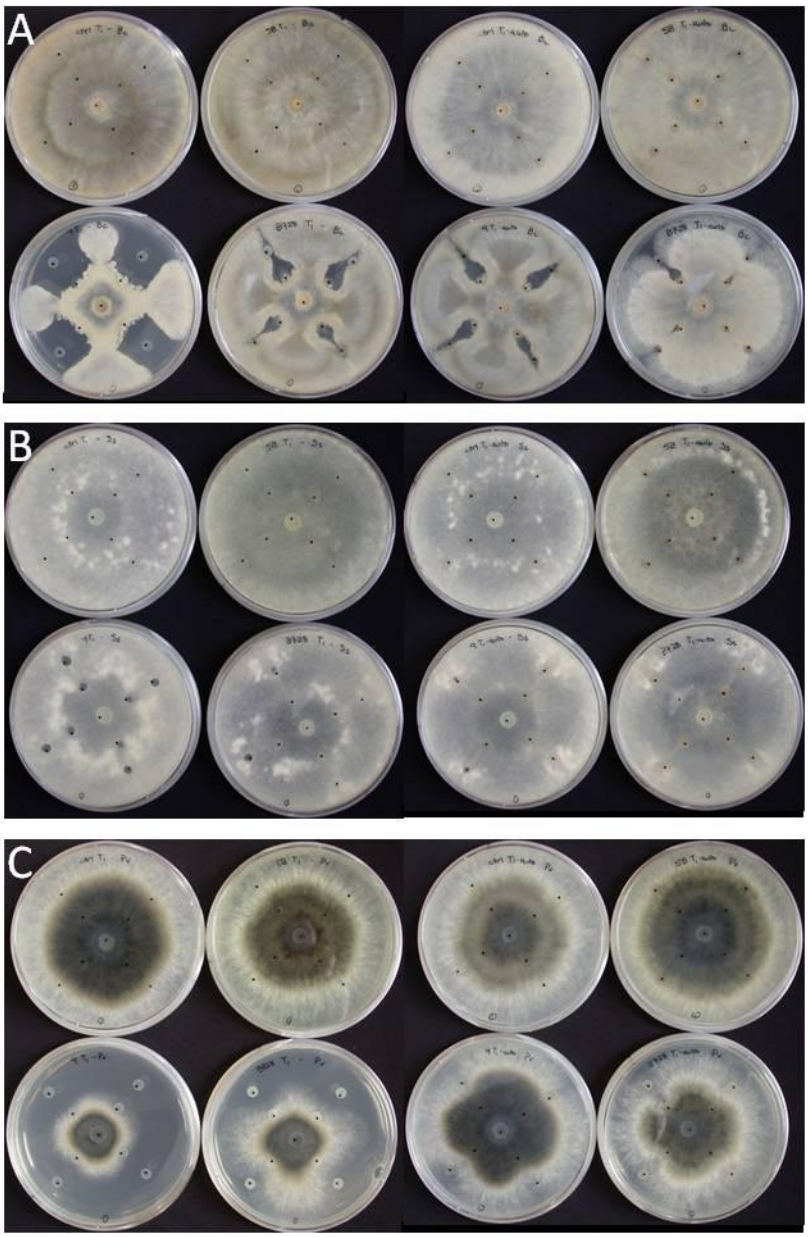

Figure A1-3. In vitro assay of inhibition of fungal growth by crude bacterial extracts and autoclaved extracts after 7 days incubation.

(A) *Botrytis*, (B) *Sclerotinia* and (C) *Phomopsis* after 7d incubation at room temperature with bacterial extracts. Top rows = PDB, 5B, PDB-autoclaved, 5B-autoclaved. Bottom rows = Pss9, B728, Pss9-autoclaved, Pss B728-autoclaved. PDB = sterile broth, 5B = *P. viridiflava* negative control, Pss B728 = positive control for syringomycin production. Bacterial cultures were grown in syringomycin-inducing medium for 6 d at RT then killed by addition of acidified acetone. Following centrifugation, supernatants were concentrated by rotary flash evaporation and extracted with butanol:water. 8 droplets of the resulting extractions were placed on plates of PDA with a fungal plug at the centre. Plates were incubated in the dark at RT.

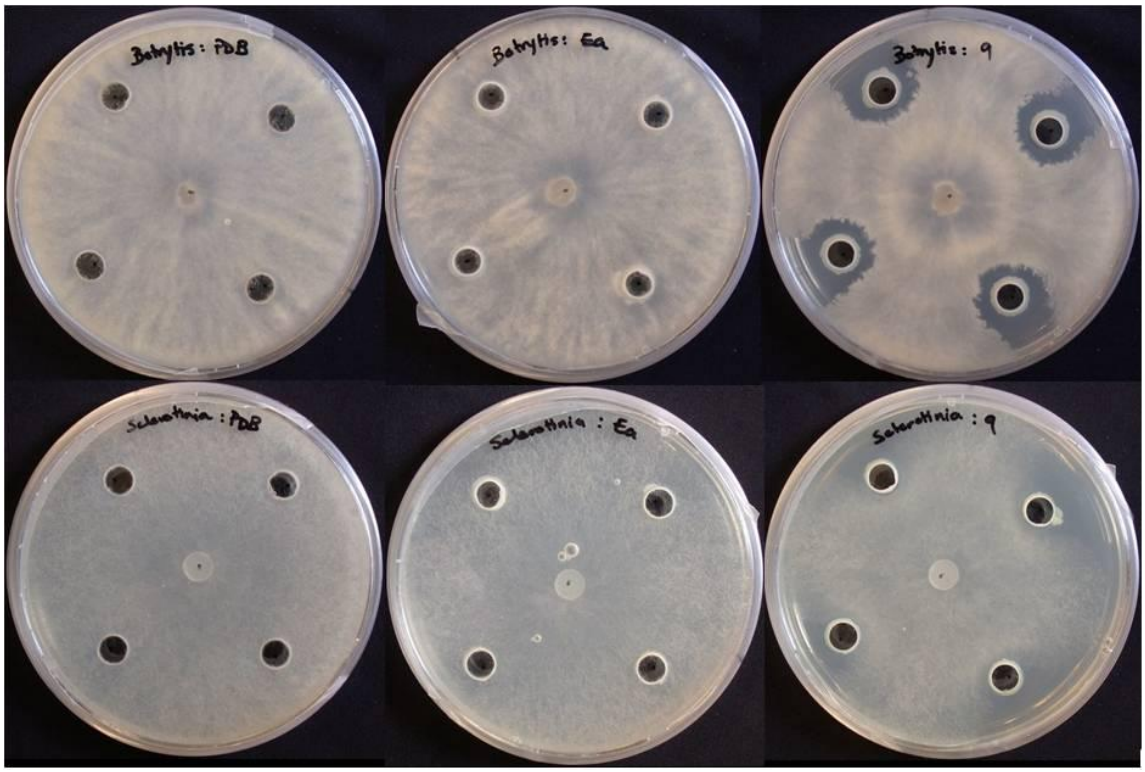

Figure A1-4. *In vitro* assay of live bacteria against *Botrytis* and *Sclerotinia* after 5 days incubation.

100 µL of sterile broth (PDB), 48-h culture of *Erwinia amylovora* (Ea), or 48-h culture of Pss9 (9) were pipetted into wells punched into PDA plates with a #3 cork borer. Fungal plugs were placed at the center of the plate and were incubated in the dark for 5 d at RT. Top row = *Botrytis* vs. (from left to right): PDB, Ea, or 9. Bottom row = *Sclerotinia* vs. (from left to right): PDB, Ea, or 9.

A2. LOPAT biochemical test results

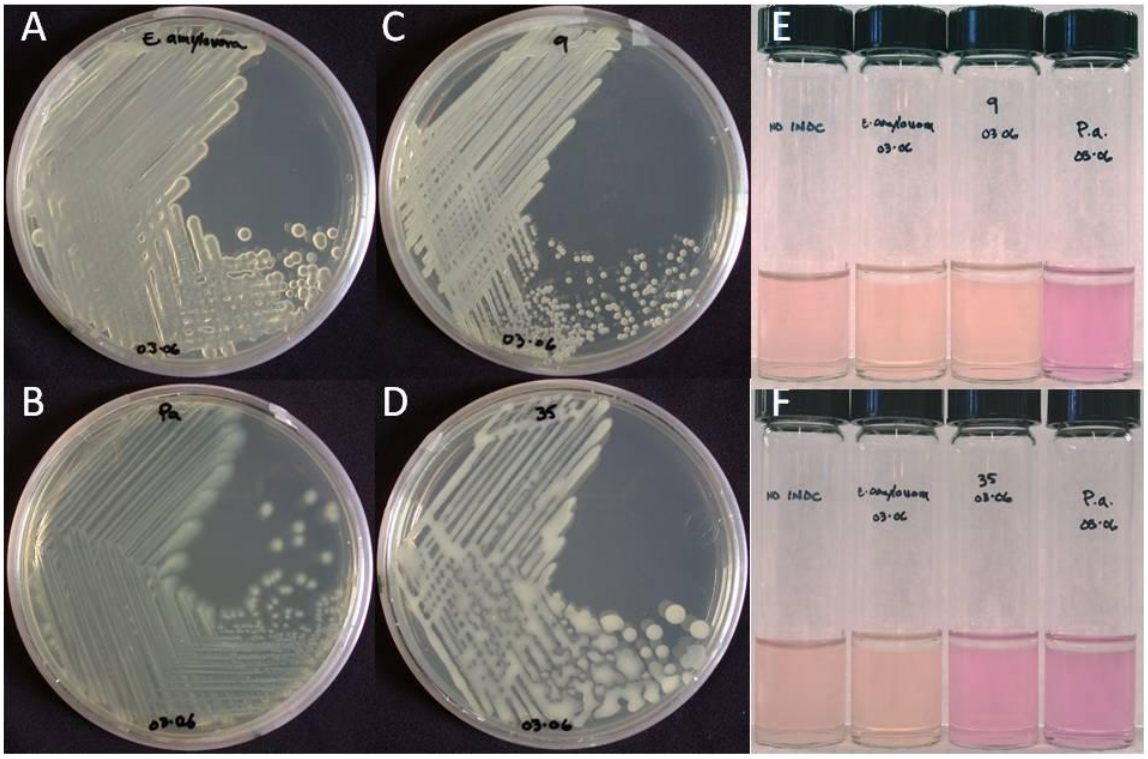

Figure A2-1. Selected LOPAT biochemical tests to determine identity of bacterial isolates collected from field tissues and comparison with type cultures.

(A-D) Levan production on NSA after 3 days incubation at RT. (E-F) Arginine dihydrolase activity under anaerobic conditions in Thornley's medium after 3 days incubation at RT. (A) *Erwinia amylovora* is levan-positive as indicated by domed, glistening colonies. (B) *Pseudomonas aeruginosa* is levan-negative. (C) Pss9 is levan-positive. (D) Isolate 35 is levan-negative but did produce slime. (E) Tubes in order from left to right are: uninoculated tube of Thornley's medium, *E. amylovora* (negative control), Pss9, and *P. aeruginosa* (positive control). (F) Tubes in the same order as in (E) but isolate 35 replaces Pss9 and shows a positive reaction.

A3. Weather variables during field studies

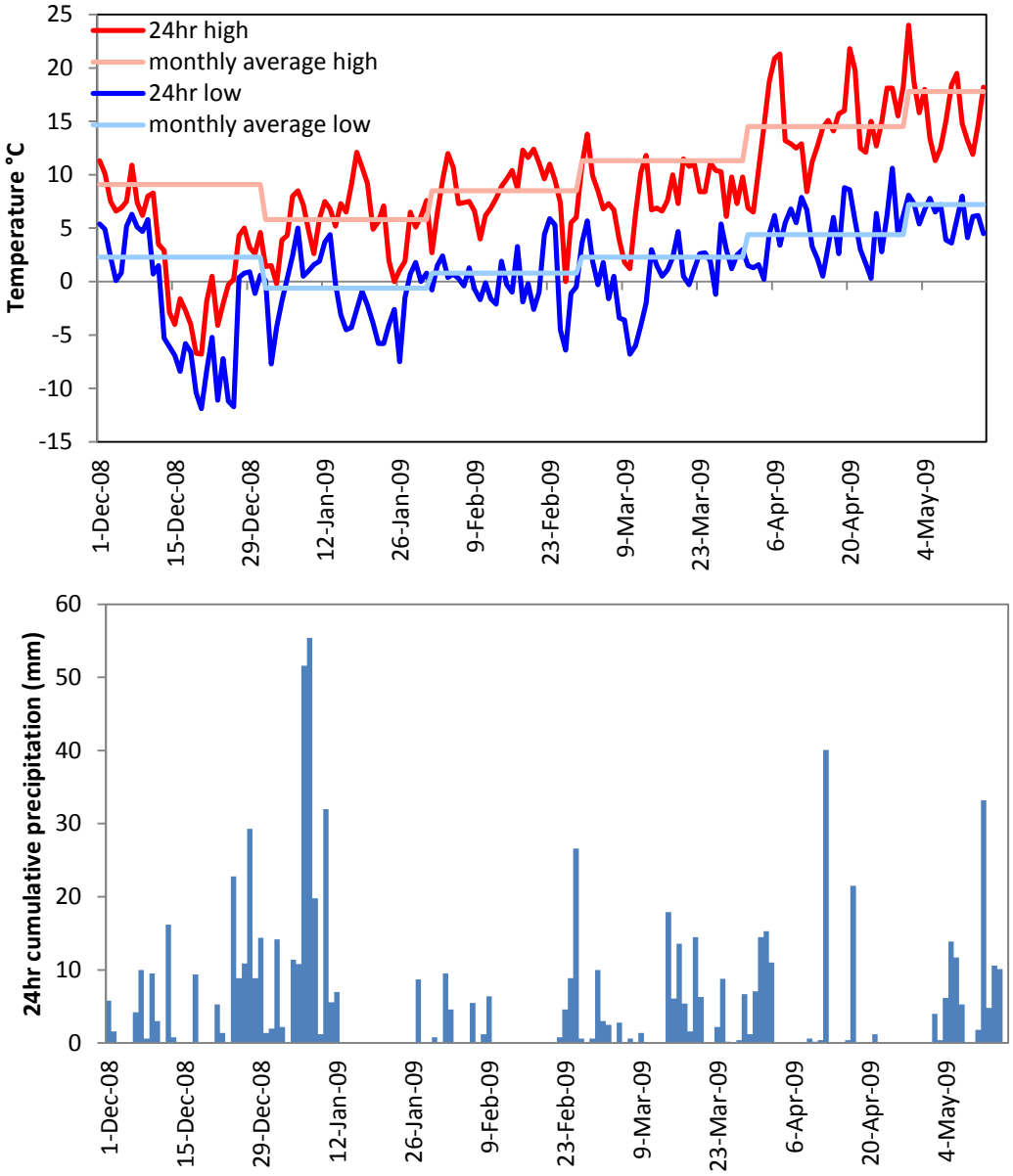

Figure A3-1. Weather variables for the duration of the first year of the field trial (2008-09).

(Top) Daily high and low temperatures at the Abbotsford airport field site from December – May with monthly averages based on 30-year data from Environment Canada's Weather Office. Lowest frequency of *Pseudomonas* recovery corresponds to lowest temperatures in December. (Bottom) Daily cumulative precipitation data.

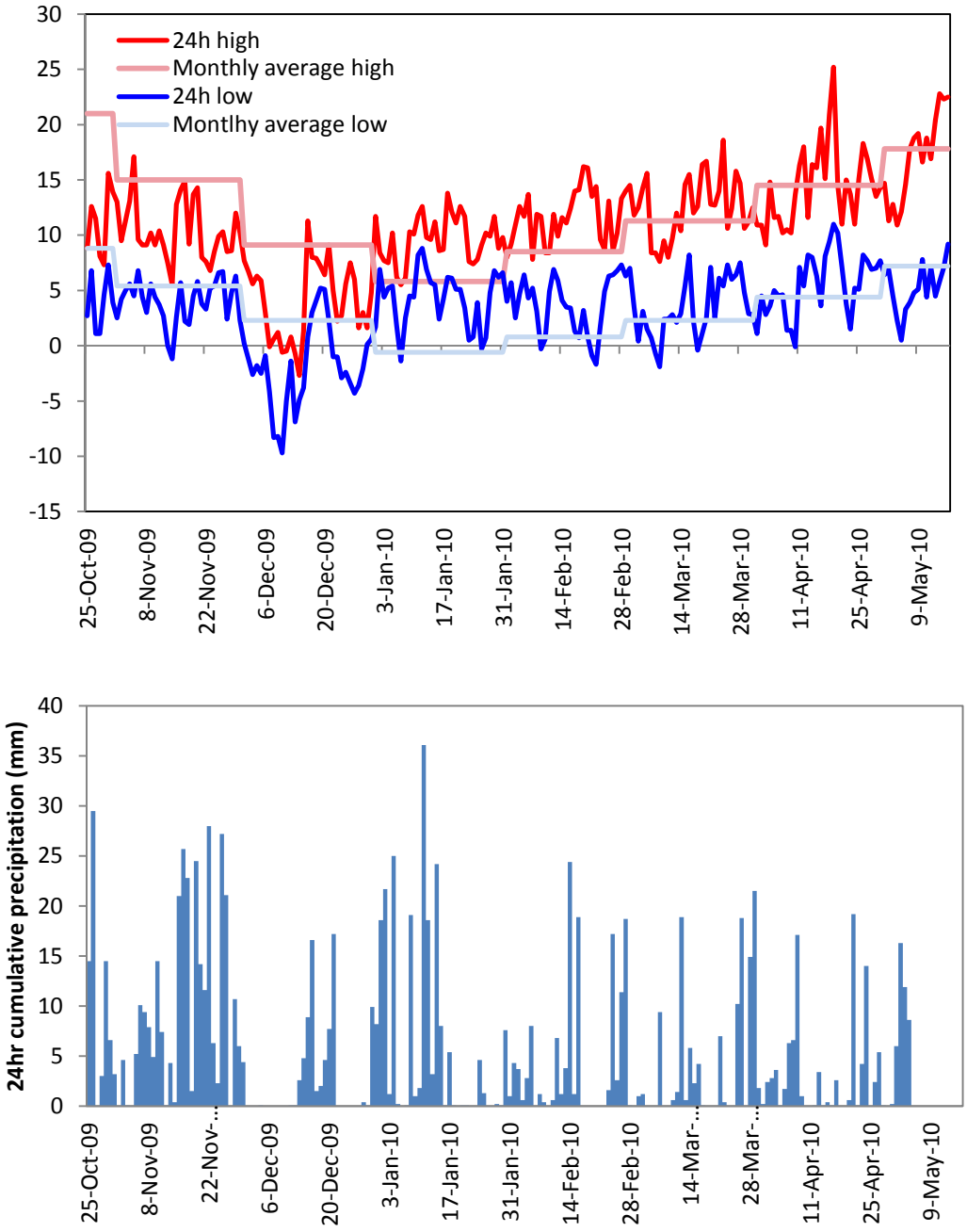

Figure A3-2. Weather variables for the duration of the second year of the field trial (2009-10).

(Top) Daily high and low temperatures at the Abbotsford airport field site from October – May with monthly averages based on 30-year data from Environment Canada's Weather Office. (Bottom) Daily cumulative precipitation data.

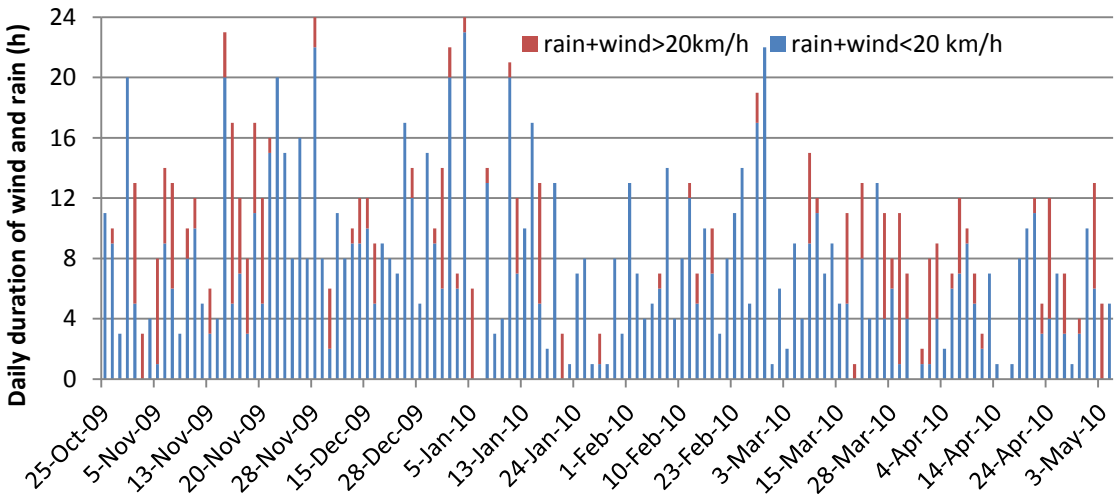

Figure A3-3. Daily hours of wind-driven rain during the second year of the field trial (2009-10).

Total bar length represents hours per day of co-occurring wind and rain, with the hours with windspeeds greater than 20 km/h in red stacked on windspeeds less than 20 km/h in blue. Gusts of wind greater than 50 km/h occurred 10 times in the period of October-November, with maximum gusts of 102 km/h from the north-northeast on Nov. 16 during a rain event. Such an event could easily disperse bacteria from inoculated plants to uninoculated plants situated downwind.

A4. Leaf Disk Assays Tabulated Results

Table A4-1. Disease ratings and number of recovered bacteria in Duke leaf discs for three trials conducted at different times of the growing season.

		Disease rating[abc]				Recovered CFU/mm^2 [ab]			
Trial[d]	Treatment	3 dpi		5 dpi		KBC		NA	
May 26: full bloom	Control	0.02 (0.02)	a	0.06 (0.03)	a†	0	a	0	a
	Pss9	0.86 (0.05)	b	1.22 (0.08)	b†	6.0×10^5 (2.4×10^5)	b	7.5×10^5 (6.8×10^5)	b
	Pss9R	0	a	0.05 (0.01)	a†	9.1×10^3 (3.6×10^3)	ab	2.2×10^4 (1.7×10^4)	ab
June 12: bloom + green berry	Control	0	a	0.03 (0.03)	a	0	a	1.0×10^{-1} (1.0×10^{-1})	a
	Pss9	0.69 (0.08)	b	1.34 (0.23)	b†	3.8×10^3 (1.6×10^3)	b	9.9×10^3 (9.6×10^2)	b
	Pss9R	0	a	0.33 (0.06)	a†	1.4×10^2 (2.3×10^1)	ab	1.3×10^3 (1.8×10^2)	ab
July 15: 50% blue berry	Control	0.30 (0.12)	a	0.29 (0.12)	a	0	a	0	a
	Pss9	0.63 (0.11)	b	0.75 (0.12)	b	5.6×10^1 (5.5×10^1)	a	3.7×10^2 (2.4×10^2)	a
	Pss9R	nt		nt					

[a] Values in parentheses represent standard error of the mean.

[b] Values followed by the same letter are not significantly different within a column. Values followed by the † have significantly different disease ratings between 3 and 5 dpi. No significant differences were found for bacterial recovery on *Pseudomonas*-selective and non-selective agars.

[c] nt = not tested

[d] Date of tissue harvest and phenological status of blueberry bushes in the field.

Table A4-2. Disease ratings and number of recovered bacteria in Elliott leaf discs for two trials conducted at different times of the growing season.

		Disease rating[abc]		Recovered CFU/mm^2 [ab]	
Trial[d]	Treatment	3 dpi	5 dpi	KB	NA
June 12: full bloom	Control	0.02 (0.01) a	0.11 (0.05) a†	0 a	0 a
	Pss9	0.78 (0.06) b	2.0 (0.15) b†	$5.6x10^1$ ($5.4x10^1$) a	$4.1x10^2$ ($3.3x10^2$) b
	Pss9R	0.12 (0.03) a	0.31 (0.05) a†	0 a	$7.9x10^0$ ($7.9x10^0$) ab
July 16: green berry	Control	0.51 (0.12) a	1.44 (0.10) a	0 a	0 a
	Pss9	0.60 (0.13) b	2.05 (0.15) b†	$5.9x10^2$ ($5.7x10^2$) b	$4.0x10^2$ ($1.9x10^2$) b
	Pss9R	nt	nt		

[a] Values in parentheses represent standard error of the mean.

[b] Values followed by the same letter are not significantly different within a column. Values followed by the † have significantly different disease ratings between 3 and 5 dpi. No significant differences were found for bacterial recovery on *Pseudomonas*-selective and non-selective agars.

[c] nt = not tested

[d] Date of tissue harvest and phenological status of blueberry bushes in the field.

A5. Disease rating scales for laboratory pathogenicity assays

Table A5-1. Disease rating scales for lilac plantlets inoculated by two methods and blueberry leaf discs.

	Lilac		Blueberry
Rating	Wounded	Dipped	Leaf disc
0	healthy	healthy	healthy
1	wound site necrotic >1 mm	small lesions visible (>1 mm)	necrosis of midvein or 10% of area necrotic
2	expanded lesion (1-2 mm)	expanded lesions (1-2 mm)	spreading necrosis or 25% necrotic
3	50% necrotic and/or wilting	large lesions (3-4 mm)	50% necrotic
4	dead	dead	75% necrotic - dead

A6. Field year 1.

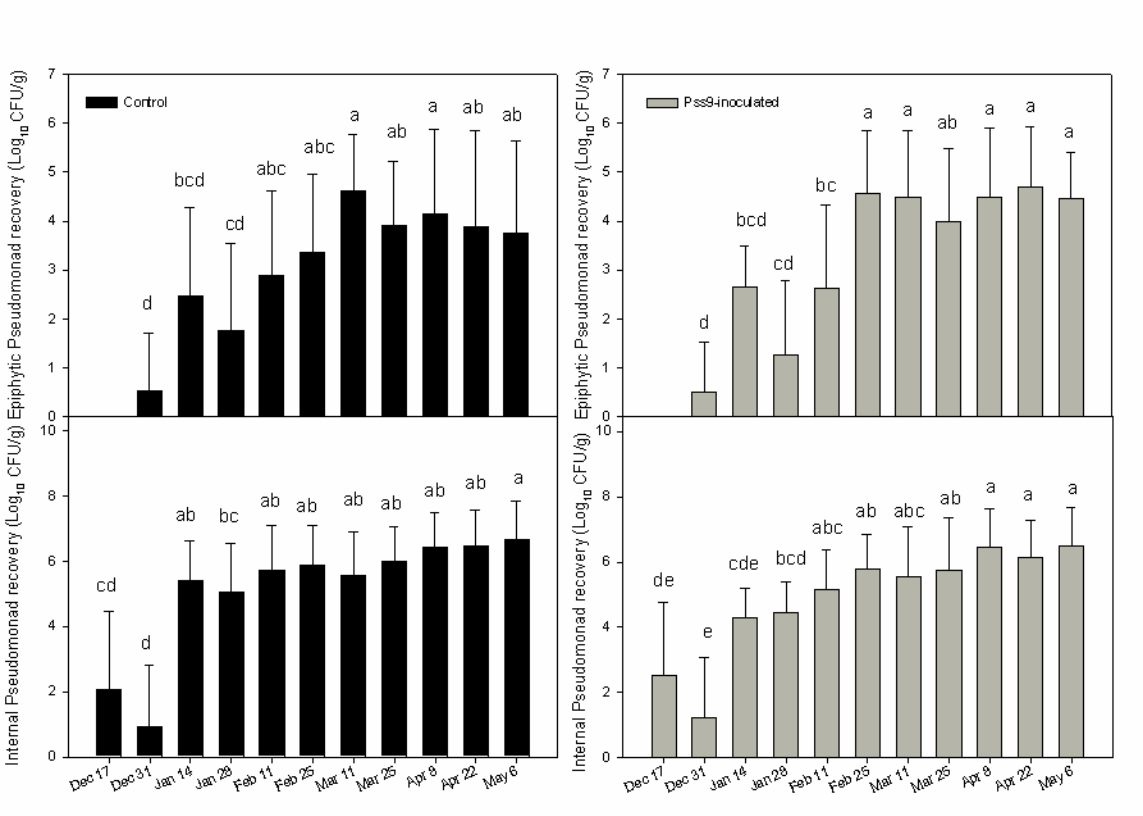

Figure A4-1. Field year 1: Differences in recovery of Pseudomonas from blueberry stems over sampling times for two treatments.

Control epiphytes (top left), control internal populations (bottom left), Pss-inoculated epiphytes (top right) and Pss9-inoculated internal populations (bottom right). Bars with the same letter are not significantly different as determined by Tukey's HSD (α = 0.05). Population progression follows the same seasonal pattern on controls and inoculated blueberry plants. Neither epiphytic nor internal populations increase significantly after late February.

Appendix B.

Ice nucleation activity of Pss9

The ability of *P. syrinage* to induce ice formation on host plants has been associated with increased disease incidence (refs). To determine whether Pss9 has ice nucleation activity, simple laboratory assays were conducted. Ice-salt (NaCl) mixtures were optimised to produce temperatures below 0°C but above -10°C. Approximately 178 g of ice chips plus 5.84 g NaCl (0.1 M) stabilized at -5°C within a few minutes. The mixture was contained in a Styrofoam cooler on the benchtop and monitored with a traceable temperature probe. Small amounts of 48-h old cultures of bacteria grown on nutrient agar were used to inoculate a sterile 15 ml screw-cap tube containing 10 ml of molecular-grade distilled water using the tip of a sterile loop. Resulting bacterial suspensions were not enumerated. Control tubes contained water only. Ice was found to form in water inoculated with Pss9 at -6°C after 10 minutes (Fig. B1-1A) whereas the pure water never froze at this temperature and required several hours at -20°C before ice crystals were initiated. The tube of pure water was accidentally agitated by opening the freezer door and this may have triggered ice formation. Isolate Pss30 nucleated ice near -4°C after 5 minutes. Differences in temperature and time required for ice nucleation may be due to the number of ice nuclei (proteins) added which may be directly related to the number of bacteria in the suspensions.

Indirect assays of Pss9's ability to nucleate ice used PCR detection of *ina* genes (Fig. B1-1B). Primer pairs for amplification of *inaZ* (Green and Warren, 1985) produced bands for isolates1, 27, 28, 29, 30, 32, 38, 46 and Pss9. Excision and purification of the band from the gel for Pss9 followed by amplification by cloning resulted in poor sequencing data. Therefore, it could not be determined whether the amplicon was actually the produce of an ice nucleation gene. PCR detection of *inaV* (Schmid *et al*., 1997) failed for most isolates tested, including Pss9.

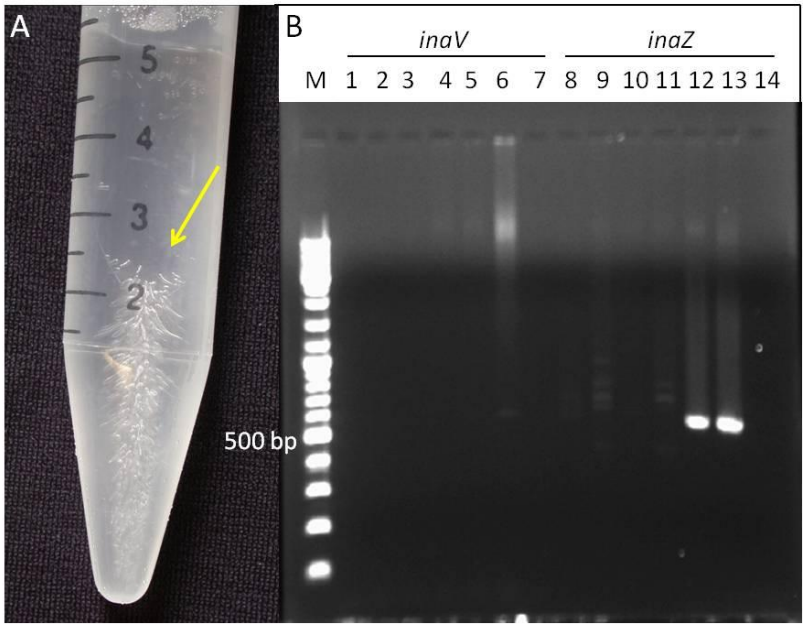

Figure B1-1. Ice nucleation activity of Pseudomonas isolates from commercial blueberry fields.

(A) Pure water inoculated with Pss9 shows ice crystal formation initiated at -6°C. Yellow arrow indicates ice development within tube of water. (B) PCR detection of *ina* genes. M is the 10 Kb O'geneRuler DNA ladder (Fermentas). Tested isolates were loaded onto the gel as follows: isolate 20B, lanes 1 and 8; isolate 21, lanes 2 and 9, isolate 24, lanes 3 and 10, isolate 25, lanes 4 and 11; isolate 27, lanes 5 and 12; isolate 28, lanes 6 and 13. Lanes 7 and 14 are PCR blanks.

Appendix C.

Copper resistance of Pss isolates in the Fraser Valley

Resistance to copper bactericides has been reported previously in the Fraser Valley (MacDonald *et al*., 2002). Initial screening for copper resistance in isolates obtained in this study was conducted in screw-cap culture tubes containing 10 ml LB medium amended with $CuSO_4 \cdot 5H_2O$ to give final concentrations of the active ingredient (copper ions) at 0 mM, 0.16 mM or 0.32 mM. These values were chosen based on previously published reports (MacDonald *et al*., 2002) and reflect zero, low, and medium application rates in fields. High rates (0.64 mM) were not tested. Duplicate tubes were inoculated with 100 µL of starter cultures, then gently agitated at 50-70 RPM on an orbital shaker and incubation was at room temperature (RT) for 48h. Afterwards, 1 mL aliquots were sampled for reading of optical density at 590 nm. Cultures were then left to incubate quiescently for 1 week at RT. Results are shown in Table C1-1. Following the quiescent incubation, 200 µL of each suspension showing turbidity was plated on *Pseudomonas*-selective medium (KB-C) and incubated at RT for 24 h. All plated suspensions produced confluent growth indicating that turbidity was not due exclusively to dead cells.

Table C1-1. Growth of Pss isolates and type culture in LB medium amended with various concentrations of copper sulfate as measured by optical density at 590 nm.

	Copper sulfate concentration in LB medium					
	0 mM		0.16 mM[a]		0.32 mM	
Isolate	48 h	7 d	48 h	7 d	48 h	7 d
Pss9	1.412	0.489	0.021	0.171	0.032	0.082
Pss30	1.417	0.549	1.037	0.024	0.029	0.694
NCPPB 281	1.168	1.147	0.0	nt	0.0	nt

[a] nt = not tested.

Further testing of isolates was conducted in triplicate using 96-well microtitre plates containing 150 µL of LB amended as previously described to give zero, low and medium rates of copper ions. Microtitre plates were inoculated with 1 µL of starter cultures and then incubated at RT with gentle agitation on a platform shaker. Plates were read with a plate scanner at 590 nm after 48 h. Results are shown in Table C1-2. None of the isolates tested were able to grow at the medium concentration of copper but all could grow at low concentrations, except for four isolates. These four strains were isolated from nursery-grown Hibiscus (H), Viburnum (L) and wild poplar (P and Q). Isolates from wild poplar would never have encountered environmental levels of copper seen in commercial blueberry fields or nurseries and would therefore never develop resistance to this bactericide. The reasons for growth at low levels but not medium levels of copper ions was not investigated. Detection of conjugative plasmids that are known to carry copper resistance operons by PCR was not successful (data not shown).

Table C1-2. Growth of bacterial isolates in LB medium amended with various concentrations of copper sulfate as measured by optical density at 590 nm.

	Copper sulfate concentration in LB medium		
Isolate	**0 mM**	**0.16 mM[a]**	**0.32 mM[a]**
1	1.497	1.272	NG
2	1.241	1.141	NG
5B	0.324	0.269	NG
9	1.428	0.005	NG
19	1.270	1.169	NG
20B	1.193	1.005	NG
21	1.303	1.053	NG
24	1.242	0.196	NG
25	1.249	1.151	NG
27	1.281	0.826	NG
30	1.315	0.939	NG
44	1.284	0.013	0.015
49	1.120	0.860	NG
50	1.245	0.687	NG
67	1.307	1.248	NG
C	1.364	0.896	NG
D	1.389	0.924	0.019
H	1.377	NG	NG
L	1.320	NG	NG
M	1.510	1.180	NG
O	1.256	1.080	NG
P	1.283	NG	NG
Q	1.219	NG	NG
P. aeruginosa	1.643	1.394	0.022

[a] NG = no growth. OD values were < 0.01

Appendix D.

Biological control as an alternative management tool for bacterial blight

The single registered chemical control for bacterial blight on blueberry is fixed copper. Although available in several formulations, the use of copper ions as the sole active ingredient in allowable bactericides raises concerns over the development of resistance. Organic growers and conventional growers who wish to use alternative methods for bacterial blight management may turn to biological controls agents, or BCAs. Generally, these are live microorganisms industrially formulated for storage, application through high-pressure sprayers and survival (short-term) in the phylloplane. Products currently available in Canada include Serenade MAX® (*Bacillus subtilis* QST 713), Bloomtime Biological™ FD (*Pantoea agglomerans* E325), Blightban C9-1 (*Pantoea agglomerans* C9-1) and BlightBan A506 (*P. fluorescens* A506). Highbush blueberry is label-listed for Serenade, but not the other products which are largely for suppression of fireblight (*Erwinia amylovora*) in pome fruit orchards.

Laboratory assays of the efficacy of these products in controlling Pss9 were conducted on agar plates and detached blueberry leaves. Copper oxychloride, streptomycin sulfate and silver nitrate were used as chemical standards for comparison. Co-plating assays were used to determine whether antibiosis is an effective mechanism in biological control of Pss and all assays used ½-strength nutrient agar (NA) as the medium. The zone of growth inhibition between products and Pss9 was measured for several methods. The first method used well plates (wells punched at the centre into which were pipetted 100 µL of each tested product) that were overspread with a suspension of Pss9. Other methods included products and Pss9 streaked in parallel (5 mm apart) and perpendicular streaks. Perpendicular streaks were examined for overgrowth of one bacterium over the other or failure to overgrow chemical products. Relative inhibition was examined by two methods: plating of Pss9 either directly on medium amended with products or as an overlay. For the overlay method, plates were spread with products and incubated for 24 h. The next day, ½-strength NA with 0.75% agar was autoclaved in tubes, cooled to approximately 45°C and amended with Pss9. The suspension was swirled to mix and then poured over the plates prepared on the first day. All plates were incubated at RT for 3-5 days. There was not sufficient time for co-culturing in liquid media although this had been planned.

For the well plates, streptomycin produced the greatest zone of inhibition (6 mm), followed by silver nitrate (1 mm) and no control from copper or Serenade (Table D1-1). Copper could not diffuse into the agar as streptomycin did and Serenade did not grow quickly enough to inhibit growth of Pss. For parallel streaking, no inhibition of Pss was detected and this may have been due to the lack of diffusion of antimicrobial substances across the 5 mm (Table D1-1). Perpendicular streaks showed that *Bacillus subtilis* (Serenade) and *Pantoea agglomerans* (Bloomtime) can overgrow Pss9 (Table D1-1). Interestingly, *P. agglomerans* was able to overgrow *B. subtilis* (data not shown). For the amended medium trials, both *B. subtilis* and copper inhibited growth of Pss9 by 100% in comparison with growth on unamended plates. For the overlay experiments, mixed cultures grew on the surface of the plates and it was not determined whether this was due to foreign contaminants, impurity of products or the natural variability within the product strains. Most colonies were not Pss9; however, in the Serenade treatment, most colonies also were not a bacillus.

Effective BCAs must be competent in colonizing the host tissue that they are presumed to protect. To determine whether any of the products tested in vitro could be extended to blueberry tissue, products were applied to surface-sterilized detached leaves. Products were prepared at the manufacturer's suggested concentration for low rate applications and were suspended in phosphate buffered saline. Leaves were dipped in the resulting solutions for two minutes. No

surfactants were used to avoid possible chemical inhibition of the BCAs.Control leaves were dipped in sterile buffer. Leaves were then placed on moistened filter paper in sterile humidity chambers and left to incubate at RT for 5 d. Leaves were then macerated in buffer for serial dilution plating to enumerate and identify any colonizing bacteria. Results are shown in Table D1-2. Pantoea agglomerans seems to be highly proficient at colonizing blueberry leaves and also can out-colonize Pss9 when inoculated at the same time. This is a very promising product for control of bacterial blight but field efficacy data are needed prior to requesting label expansion to include highbush blueberry.

Table D1-1. In vitro assays of chemical and biological control agents against Pss9.

	Zone of inhibition[a] (mm)		Overgrowth[b] (+/-)	Relative inhibition (%)[c]		
Product	Well plate	Parallel streaks	Crossed streaks	Amended medium	Amended overlay[d]	Broth co-culturing[e]
Copper hydroxychloride	0	0	n/a	100	80?	nt
Streptomycin sulfate	6	0	n/a	nt	nt	nt
Silver nitrate	1	0	n/a	nt	nt	nt
Serenade Max	0	0	++	100	50?	nt
Bloomtime	nt	0	nt	nt	70?	nt
Blightban C9-1	nt	0	++	nt	nt	nt
Blightban A506	nt	0	nt	nt	nt	nt
Control (no product)	0	0	n/a	0	0	nt

[a] nt = not tested

[b] + = some overgrowth of Pss9 by product, ++ = lots of overgrowth by product, +++ = absolute inhibition of Pss9 by product

[c] Relative inhibition calculated as percent average Pss9 growth on amended medium vs. average Pss9 growth on control plates.

[d] Possible contamination by foreign bacteria, data questionable.

[e] Co-culturing in liquid medium was not performed due to time constraints.

Table D1-2. Colonization of detached blueberry leaves by BCAs and Pss9.

	CFU/g fw	
Product/bacterium	Trial 1	Trial 2
Control	0	0
Copper hydroxychloride	1.1×10^4	0
Pss9	6.3×10^4	7.4×10^5
P. agglomerans (Bloomtime)	1.68×10^4	6.9×10^5
B. subtilis (Serenade)	nt	3.27×10^5
Pss9 + *P. agglomerans*	nt	6.7×10^5 P.ag / 0 Pss9
Pss9 + *B. subtilis*	nt	1.6×10^6 Pss9 / 0 B.s.
Pss9 + *E. coli*	nt	3.08×10^5 mixture